CORRELATION OF ACETYLCHOLINE RECEPTOR ANTIBODY TITRE WITH DISEASE ACTIVITY MEASURED BY PEMPHIGUS DISEASE AREA INDEX (PDAI) AND DESMOGLEIN ANTIBODY TITRE IN PEMPHIGUS PATIENTS IN A TERTIARY CARE CENTRE, SOUTH INDIA

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE RULES AND REGULATIONS FOR THE M.D. BRANCH XX DERMATOLOGY, VENEREOLOGY AND LEPROSY EXAMINATION OF THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY TO BE HELD IN APRIL, 2016

CERTIFICATE

This is to certify that the dissertation entitled **"Correlation of acetylcholine receptor antibody titre with disease activity measured by PDAI and desmoglein antibody titre in pemphigus patients"** is a bonafide original work of Dr.L.Rosemary.

This study was undertaken at Christian Medical College and Hospital from October 2014 to August 2015 under my direct guidance and supervision, in partial fulfillment of the requirement of the award of the M.D degree (Branch XX) in Dermatology, Venereology and Leprosy of the Tamil Nadu Dr. M.G.R Medical University.

Guide	Principal	Head of the Department
Dr. Dincy Peter, Professor, Department of Dermatology, Venereology and Leprosy, Christian Medical College, Vellore	Dr. Alfred Daniel, Christian Medical College, Vellore	Dr. Renu George, Professor and Head, Department of Dermatology, Venereology and Leprosy, Christian Medical College, Vellore

DECLARATION

I hereby declare that this M.D dissertation entitled **"Correlation of acetylcholine receptor antibody titre with disease activity measured by PDAI and desmoglein antibody titre in pemphigus patients"** is the bonafide work done by me under the guidance of Dr. Dincy Peter, Professor, Department of Dermatology, Venereology and Leprosy, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

Dr. L. Rosemary,
Postgraduate resident,
Department of Dermatology, Venereology and Leprosy,
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Last but not the least my wholehearted thankfulness to all the patients for their participation.

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Antibodies to Acetylcholine receptors and disease activity in pemphigus.
Dr. L. Rosemary, PG Registrar, Dr. Dincy Peter, Dr. Susanne Pulimood,
Dr.Victoria Job, Biochemistry, Dr. Leni George, Dr. Anu Anna George, Dr.Nirmal,
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Dear Dr. L. Rosemary,

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1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

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Dear Dr. L. Rosemary,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Antibodies to Acetylcholine receptors and disease activity in pemphigus." on August 4th 2014.

The Committees reviewed the following documents:

1. IRB Application format
2. Curriculum Vitae' of Drs. L. Rosemary, Dincy Peter, Susanne Pulimood, Victoria Job, Leni George, Anu Anna George, Nirmal.
3. Informed Consent form (English, Tamil, Hindi & Telugu)
4. Information Sheet (English, Tamil, Hindi & Telugu)
5. No of documents 1-4

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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 50,000/- INR (Rupees Fifty Thousand only) will be granted for 1 year.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
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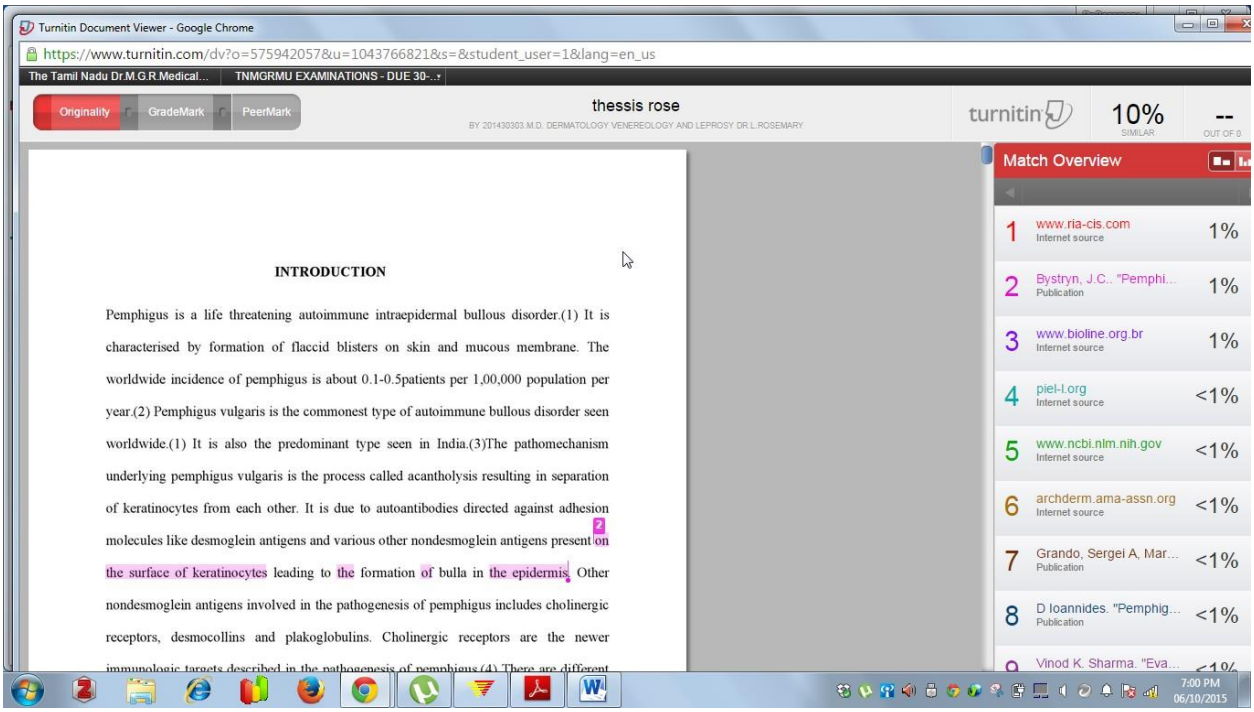
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INTRODUCTION
Pemphigus is a life threatening autoimmune intraepidermal bullous disorder.(1) It is characterised by formation of flaccid blisters on skin and mucous membrane. The worldwide incidence of pemphigus is about 0.1-0.5patients per 1,00,000 population per year.(2) Pemphigus vulgaris is the commonest type of autoimmune bullous disorder seen worldwide.(1) It is also the predominant type seen in India.(3)The pathomechanism underlying pemphigus vulgaris is the process called acantholysis resulting in separation of keratinocytes from each other. It is due to autoantibodies directed against adhesion molecules like desmoglein antigens and various other nondesmoglein antigens present on the surface of keratinocytes leading to the formation of bulla in the epidermis. Other nondesmoglein antigens involved in the pathogenesis of pemphigus includes cholinergic receptors, desmocollins and plakoglobulins. Cholinergic receptors are the newer
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ABSTRACT

TITLE OF THE ABSTRACT: Antibodies to acetylcholine receptor antibody titre, anti desmoglein antibody titre and disease activity in pemphigus patients.

DEPARTMENT: Department of Dermatology , Venereology and Leprosy.

NAME OF THE CANDIDATE: L.Rosemary

DEGREE AND SUBJECT: M.D. Dermatology, Venereology and Leprosy

NAME OF THE GUIDE: Dr. Dincy Peter

OBJECTIVES: Correlation of acetylcholine receptor antibody titre with disease activity measured by PDAI and desmoglein antibody titre in pemphigus patients

METHODS: In this study, 77 Pemphigus vulgaris patients both naïve and on treatment were recruited after obtaining their consent. Data on demographic profile, duration of illness, subtype of pemphigus vulgaris, treatment details were recorded. At the time of initial presentation the disease severity was evaluated by pemphigus disease area index score. Anti desmoglein antibody titre and acetycholine receptor antibody titre was estumates using ELISA kit and then compared with PDAI score. Pearsons product moment correlation coefficient was used to correlate PDAI score with AChRAb titre and anti desmoglein antibody titre.

RESULTS: The mean age of the patient was 46yrs and there was female preponderance with a male:female ratio of 1:1.2 . Mucocutaneous subtype (75%) of pemphigus was predominant in the study group with majority (40%) of them with raised titres of both Dsg1 and Dsg3. In mucosal type of pemphigus, 10% of them had raised titres of anti Dsg3 antibody but Dsg1 antibody positivity was not seen. Majority (55%) of them had mild disease. About 17 patients (22%) of study population had positive titres of AChRAb titre. There was significant statistical correlation of PDAI score with anti Dsg1 and Dsg3 antibody titre.(p-value - <0.01). There was no statistical significant correlation of AChRAb titre with PDAI score and anti desmoglein antibody titre.

TABLE OF CONTENTS

ABBREVIATIONS

AChRAb	- Acetylcholine receptor antibody
ATP	-Adenosine triphosphate
BSA	- Body surface area
Dsg1	- Desmoglein 1
Dsg3	- Desmoglein 3
DIF	- Direct immunofluroscence
ELISA	- Enzyme linked immunosorbent assay
EGF	- Epidermal growth factor
JPDSS	-Japenese pemphigus disease severity score
MAPK	- Mitogen activated protein kinase
PDAI	-Pemphigus disease area index
SERCA	-Sarcoendoplasmic reticulum calcium

INTRODUCTION

Pemphigus is a life threatening autoimmune intraepidermal bullous disorder(1). It is characterised by formation of flaccid blisters on skin and mucous membrane. The worldwide incidence of pemphigus is about 0.1-0.5 patients per 1,00,000 population per year(2). Pemphigus vulgaris is the commonest type of autoimmune bullous disorder seen worldwide(1). It is also the predominant type of autoimmune bullous disorder seen in India(3–6). The pathomechanism underlying pemphigus vulgaris is the process called acantholysis resulting in separation of keratinocytes from each other. It is due to autoantibodies directed against adhesion molecules like desmoglein antigens and various other nondesmoglein antigens present on the surface of keratinocytes leading to the formation of bulla in the epidermis. Other nondesmoglein antigens involved in the pathogenesis of pemphigus includes cholinergic receptors, desmocollins and plakoglobulins. Cholinergic receptors are the newer immunologic targets described in the pathogenesis of pemphigus(6). There are different types of cholinergic receptors present on the surface of keratinocytes. Endogenously secreted acetylcholine activates these cholinergic receptors and helps to maintain the shape and adhesion of keratinocytes. These cholinergic receptors were found at the site where acantholysis occurs, thereby proving their role in regulation of adhesion of keratinocytes(7). The pathogenesis of pemphigus is unclear and still under intense research.

The disease severity varies from mild to life threatening disease and thus it is necessary to categorise the disease. There are various scoring system proposed to assess the severity

of disease. Of these, pemphigus disease area index (PDAI) is highly a validated, reliable, reproducible scoring system with higher intra-rater reliability in comparison to autoimmune bullous skin disorder intensity score(ABSIS)(8). It measures the disease severity over trunk, extremities, scalp and mucosa.

The desmoglein antibody titres were found to correlate with disease severity in pemphigus vulgaris(9). Daneshpazhooh et al. in his study demonstrated that Dsg ELISA is a useful tool to assess disease severity and to monitor disease activity. He also proved the correlation of clinical phenotype of pemphigus patients with desmoglein antibody profile. He found that anti Dsg1 titre correlated with severity of cutaneous involvement and anti Dsg3 titre correlated with severity of oral mucosal involvement. But in occasional cases he noticed discordance between clinical phenotype and desmoglein antibody profile. This discrepancy was postulated to be due to genetic variation or presence of minor antigens which is involved in the pathogenesis of pemphigus(10). These minor antigens includes nondesmoglein antigens like cholinergic receptors. Sanchez et al. correlated the acetylcholine M3 receptor antibody titre with disease severity measured by body surface area (BSA) and compared with Dsg3 antibody titre. He observed that there is mild rise in acetylcholine receptor antibody titre and it correlated well with disease severity and Dsg3 antibody titre at the initial presentation and also during follow up(11). The mainstay of treatment for pemphigus includes corticosteroids which has reduced the mortality from 90%to 10%. Grando and Dahl observed that antiacantholytic effect of cholinomimetics on keratinocytes in vitro(12).

This discovery unraveled a search for nonhormonal drug in the treatment of pemphigus to ameliorate side effects of steroid.

There are not many studies looking at acetylcholine receptor antibody titre and its correlation with disease activity of pemphigus and there are no published Indian studies. Hence, we proposed to conduct a study in pemphigus patients to correlate the disease activity as measured by PDAI scoring with cholinergic receptor antibody titre and with anti Dsg1 and anti Dsg3 antibody titre.

AIMS AND OBJECTIVES

Aim:

To determine the correlation of disease activity with titres of antibodies against acetylcholine muscarinic (M3) receptors in patients with pemphigus.

Objectives:

Primary objective:

1) To estimate the prevalence of acetylcholine receptor antibodies in patients with pemphigus.
2) To correlate the serum anti acetylcholine receptor antibody titres with clinical disease activity as measured by the pemphigus disease area index (PDAI) in patients with pemphigus.

Secondary objective:

To compare the values of the serum anti acetylcholine receptor antibody titres with anti desmoglein antibody titres in patients with pemphigus.

REVIEW OF LITERATURE

Introduction:

Pemphigus vulgaris is an autoimmune epidermal bullous disease that results in blistering of the skin and mucosal surfaces(13).The word pemphigus is derived from the Greek word, *pemphix* meaning blister or bubble(2). In pemphigus patients there is production of autoantibodies which are directed against desmoglein antigens on the surface of keratinocytes. This leads to loss of their cellular attachment and separation from one another forming blisters within the epidermis(13). Pemphigus has varied clinical manifestations which is based on the variation in the distribution of implicated antigens in different layers of epidermis and in different regions of the body(2). The diagnosis of pemphigus is based on the clinical manifestations (flaccid blisters and erosions on skin and oral mucosa), histopathology (epidermal acantholysis), and direct immunofluorescence (demonstrates IgG and C3 deposition in the intercellular region of epidermis in a fish net pattern)(2). The pathogenic circulating antibodies in pemphigus patients are of IgG type and is directed against desmosomal cadherins desmoglein 1 and desmoglein3. These desmoglein antibody titres are measured by ELISA method which is a specific and sensitive test to diagnose pemphigus vulgaris. According to Dr.Grando and Nguyen, apart from desmoglein antibodies, there may be other additional antibodies against cholinergic receptors which are involved in the pathogenesis of pemphigus vulgaris(14). Grando proposed a list of autoantigens involved in the pathogenesis of pemphigus. These autoantigens include desmocollins, plakoglobulin, BP180 and cholinergic receptor molecules like α3 AChR, α9 AChR, pemphaxin and other annexins, FcϵRIα(12). The significance of antibodies against different cholinergic receptors involved in the pathogenesis of pemphigus is yet to be proven.

Epidemiology:

Pemphigus vulgaris is a disease of the middle age group and affects all races and both sexes. It rarely affects children. Pemphigus vulgaris accounts for around 70% of all cases of pemphigus occurring worldwide and may be the commonest autoimmune blistering disease in Eastern countries, such as India, Malaysia, China and the Middle East(15). There is increased susceptibility of the Jewish race, especially Ashkenazi Jews to pemphigus vulgaris and has a link to HLA class II alleles(16). Pemphigus vulgaris is commoner in Indians of South Africa, than in Black or Caucasian races. It is less common in the West(15).

Epidemiology, Indian scenario:

Epidemiology of pemphigus in India has shown a different trend in comparison to Western literature(17). One Indian study have shown an early onset of disease i.e. onset below 40 years of age in majority of pemphigus patients(18). This in contrast to the studies from other parts of the world where the age of onset has been recorded as between 40yrs and 60yrs. Among the dermatology outpatient attendees, the incidence of pemphigus was found to vary between 0.09% to 1.8%(3)(18). In Thrissur district of Kerala, southern India the incidence of pemphigus was assessed based on a questionnaire survey and was found to be 4.4 million per population per year.

Aetiology:

Pemphigus is a genetically predisposed disease. Certain class II major histocompatibility complex (MHC) antigens have a markedly increased frequency in pemphigus vulgaris

patients. HLA class II alleles DRB1*0402, DRB1*1401 and DQB1*0302 are seen in Caucasians and DRB1*14 and DQB1*0503 in Japanese. MHC class II alleles encodes cell surface molecules and they are necessary for antigen presentation to immune system. Therefore it is hypothesized that these alleles helps in the presentation of the desmoglein 3 peptide to T cells. Certain peptides of desmoglein 3 is predicted to fit into DRB1*0402 peptide binding pocket on T cells and stimulates T cells thereby proving the hypothesis. Pemphigus autoantibodies against desmoglein antigens are of IgG isotype. First degree relatives of pemphigus patients have higher incidence of circulating anti- desmoglein antibodies. Pemphigus patients are at greater risk of developing certain diseases with immunological disturbances like Thymoma and Myasthenia gravis. Certain triggering factors of the disease includes pesticides, drugs, infections, certain foods containing tannins, phenols and thiols, hormones (disease exacerbation during pregnancy) ultraviolet radiation and stress(19).

Pathogenesis:

All patients of pemphigus vulgaris have circulating and skin-fixed autoantibodies directed against keratinocyte cell-surface antigens called desmosomes(2). In most cases the triggering factor of intercellular autoantibodies against desmosomes is unknown. But in some individuals it is known to be triggered by drugs(13). The most important predisposing factor is HLA association(2). It was in 1964 that Beutner and Jordan made the discovery of circulating IgG antibodies against keratinocyte surface antigens(20). And in 1980 the pathogenicity of these antibodies was proved. These antibodies when passively administered to neonatal mice induced acantholysis(21)(22). They have also

been found to be capable of inducing acantholysis in organ cultures of human skin(23). The placental transfer of these autoantibodies to the newborn babies born from mothers of active pemphigus vulgaris temporarily produced pemphigus like lesions(24). All these above evidence points towards the pathogenicity of these intercellular desmoglein antibodies. These pathogenic circulating intercellular desmoglein antibodies are diagnostic of pemphigus vulgaris and its titres correlated well with disease activity(9). And they are found in 80% of patients with active disease. There is evidence of presence of tissue fixed intercellular antibodies in lesions and adjacent healthy skin in about 90% of patients. Pemphigus vulgaris autoantibodies are usually of IgG type but at times IgM, IgA, and the complement protein C3 might also be present. IgG4 is the predominant subtype of IgG seen in active pemphigus(25). The well identified keratinocyte antigens are desmosomal molecules called desmosomal cadherins, desmoglein3 (Dsg3) of 130KDA and desmoglein1 (Dsg1) of 160 KDA molecular weight.

Desmosomes:

Desmosomes are discoid intercellular junctions of diameter 0.2 to 0.5micromteter. It consists of two electron dense plaque in each of the two adjacent cells and an intercellular cleft which separates the cells(26). These electron dense plaque consists of outer dense and inner less dense plaque which is anchored to the intermediate filament cytoskeleton. Adaptor proteins of aramidallo and plakin families anchor desmosomal cadherins to intermediate filament cytoskeleton(26). Desmosomal cadherins are transmembrane glycoproteins belonging to cadherin superfamily which are calcium dependant. The

intercellular adhesive interface is formed by desmosomal cadherins. The structure of desmosome is depicted in figure 1.

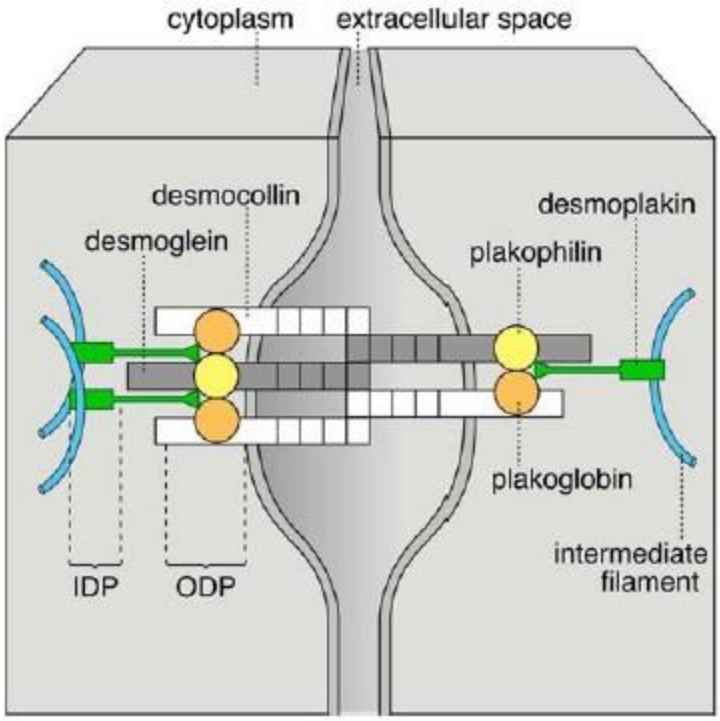


Figure 1 Molecular model of desmosome(26).

Pemphigus antibodies binds to the aminoterminal of the extracellular domain of the desmosomal cadherin(26). On binding of antibodies to desmosomal cadherins, there is increase in intercellular space and decrease and eventual disappearance of desmosome leading to rounding up of cells and detachment from one another without cell death. This process is called acantholysis(13). The process of loss of adhesion between keratinocytes known as acantholysis is the basic abnormality found in pemphigus vulgaris. Primarily there is dissolution of intercellular adhesion substance resulting in separation of desmosomes(13).The process of acantholysis ultimately leads to the formation of bulla

within the epidermis. Pemphigus antibodies do not require complement to induce blisters in the skin.

There are various proposed mechanisms of acantholysis described in pemphigus vulgaris. The exact process by which acantholysis occurs in pemphigus vulgaris is still unclear. Following are the various hypothesis concerning the pathogenesis of pemphigus vulgaris.

Mechanism of acantholysis:

1) *Plasminogen activation* - Activation of plasminogen activator leads to the formation of plasmin which induces cell adhesion dissociation(2)(26).
2) *Stearic hindrance* - It is the direct inhibition of aminoterminal of extracellular domain of demosomal cadherins by pathogenic pemphigus antibodies(2)(26)(27). The extracellular domain of desmosome is involved in transinteraction of desmosomal cadherins. However, desmosomal separation is not the primary event but collapse of cytoskeleton and shrinkage of keratinocyte precedes acantholysis.
3) *Phosphorylation of keratinocytes* - Another hypothesis is that binding of pemphigus vulgaris antibody to the antigen activates a variety of intracellular signaling pathways leading to phosphorylation of keratinocyte proteins which leads to cellular dissociation(15). P38 mitogen activated protein kinase (P38MAPK) inhibitors prevents acantholysis in mice.
4) *Apoptosis* – An earlier hypothesis had mentioned apoptosis of keratinocytes via FAS ligand pathway resulting in acantholysis. This was later disproved in that apoptosis was not a prerequisite but occurs secondary to acantholysis(28)(29).

5) *Basal cell shrinkage hypothesis* – Pathogenic autoantibody in pemphigus vulgaris binds to the receptor on keratinocytes and triggers a series of signal transduction pathways thereby resulting in rupture of cytoskeleton and collapse and shrinkage of basal keratinocytes while the keratinocytes in the suprabasal layer remain intact(29).

6) *Apoptolysis* – This is the recent theory behind the pathogenesis of skin blistering in pemphigus. It links apoptotic pathway to suprabasal acantholysis and basal cell shrinkage resulting in tomb stone appearance of basal cells(29–31). It constitutes five consecutive steps(30).

   a) Pemphigus autoantibodies binds to its antigens (desmoglein and nondesmoglein antigens) on the keratinocyte cell surface. b) Increase in intracellular calcium, activation of cell death cascades and activation of EGF receptor, Src, mTOR, p38 MAPK and other intracellular signaling elements. c) Early acantholysis due to shrinkage of basal cells which is due to collapse and retraction of tonofilament and clumping of tonofilament in the perinuclear region by executioner caspases. d) dissociation of desmosomal adhesions occurs due to phosphorylation of desmosomal molecules and cleavage of cytoplasmic tail of desmoglein by the same cell death enzymes. This inside out responses leads to internalization of extra desmosome while the established desmosomes continues to link the shrinking basal keratinocytes. e) Advanced acantholysis leads to continued degradation and cleavage of cellular protein by the same enzymes resulting in complete separation of desmosomes by shear forces from the collapsing cell thereby stimulating

secondary antibody production against the desmosomes. f) Rounding up and death of acantholytic cells. There is irreversible damage to the mitochondria and nuclear proteins(30).

The process of apoptolysis is depicted in the figure 2 and figure 3 given below.

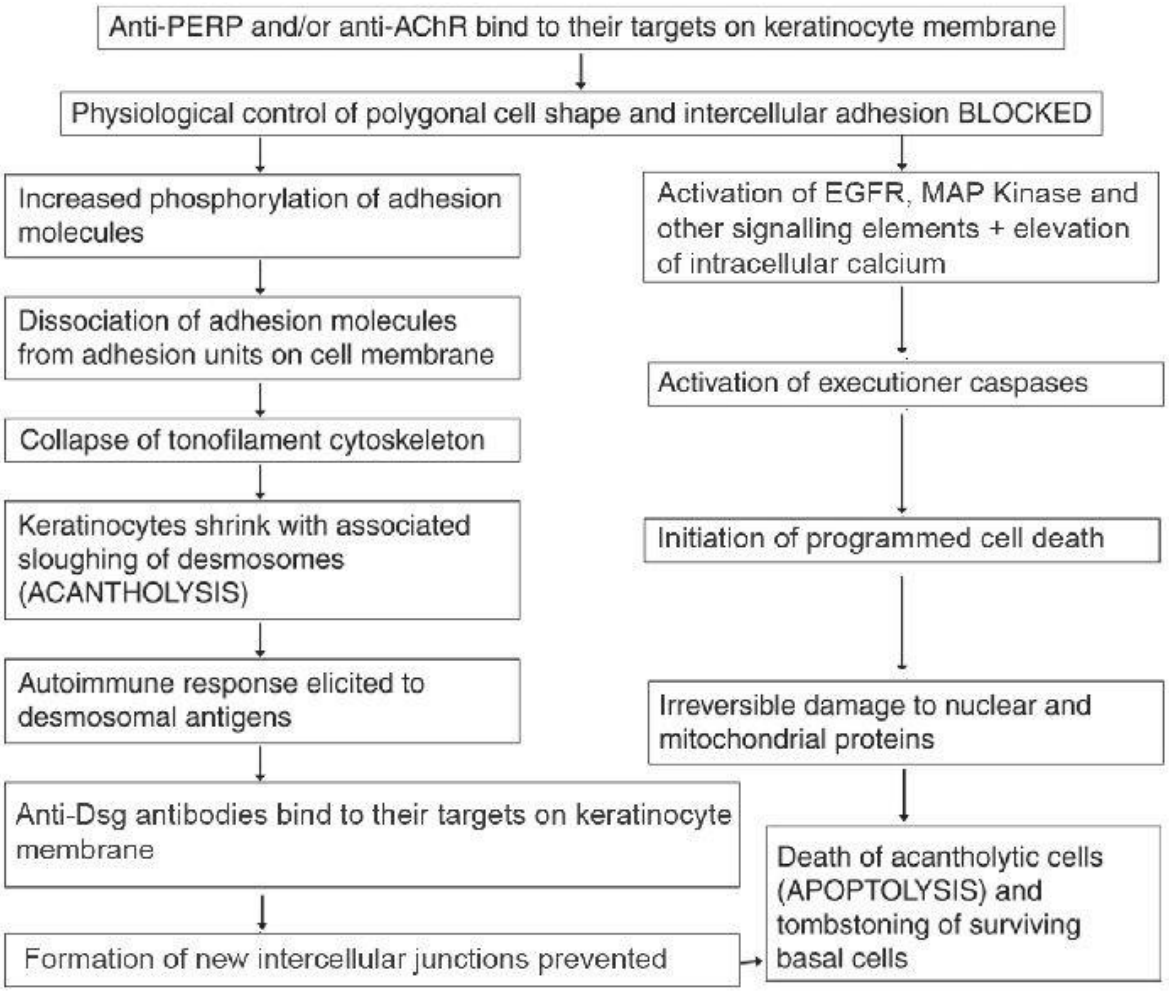


Figure 2 Current concept of acantholysis. PERP – peripheral myelin protein, EGF – epidermal growth factor, MAP – mitogen activated protein kinase(31)(32).

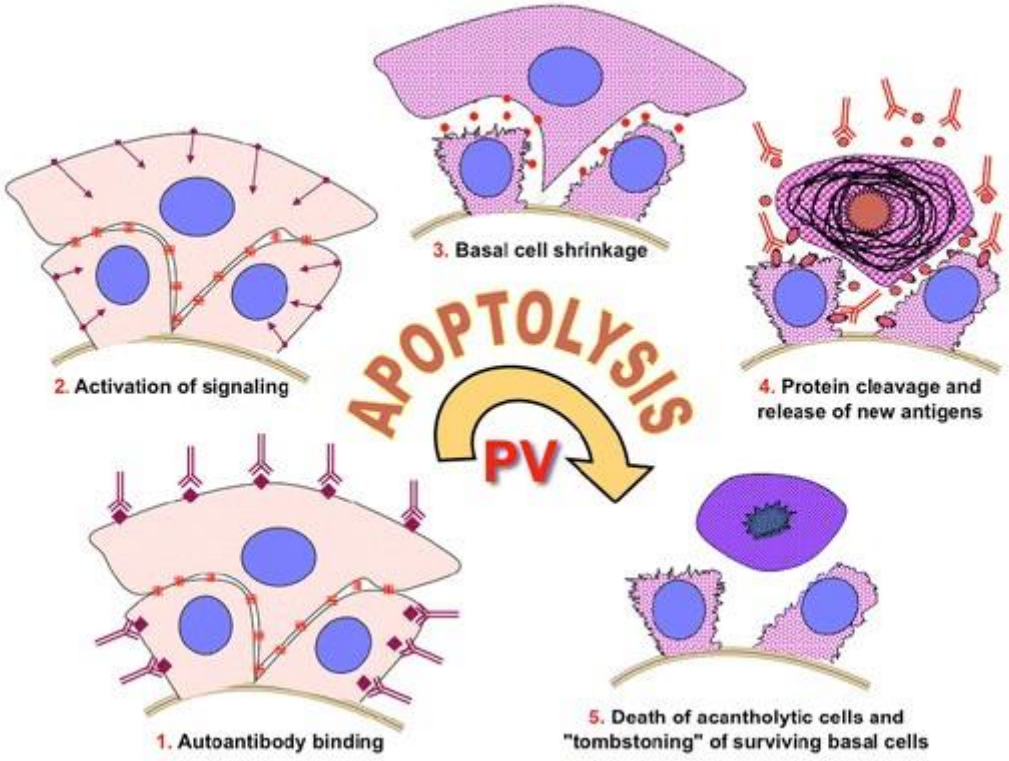


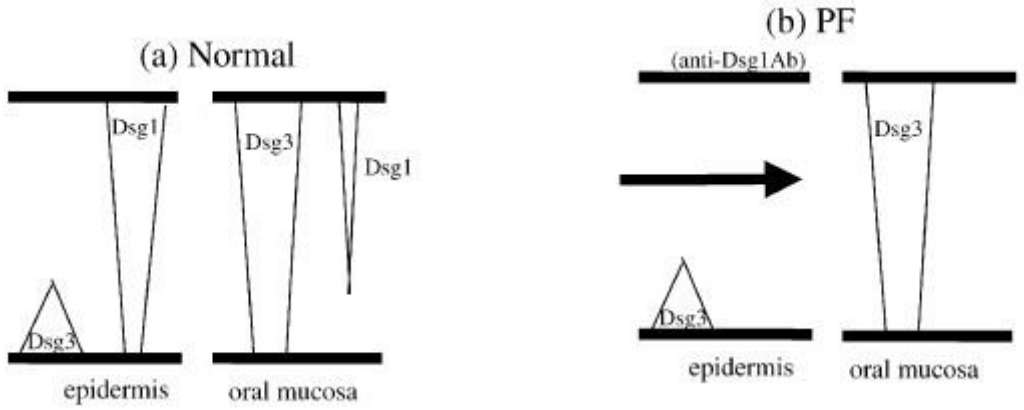
Figure 3 Hypothetical scheme of apoptolysis in pemphigus vulgaris(32).

In pemphigus vulgaris, the pathogenic antibodies IgG is targeted against various types of keratinocyte surface antigens leading to the formation of suprabasal bulla by the process of apoptolysis .

The different clinical phenotype noted in patients of pemphigus vulgaris and pemphigus foliaceous is explained by desmosomal compensation theory. Mahoney et al. have confirmed desmoglein compensation hypothesis by showing that in wild type neonatal mice both anti Dsg 3 and anti Dsg1 antibodies are required to induce skin blisters(33).

Desmosomal compensation theory:

Desmoglein 3 is present predominantly over suprabasal epidermis and is less in superficial epidermis whereas Dsg1 is distributed throughout epidermis but more over superficial layers of epidermis. In oral mucosa Dsg3 is distributed throughout epidermis while Dsg1 is poorly distributed. These desmogleins are found to compensate the functional loss of other desmoglein. This desmoglein compensation theory explains why pemphigus vulgaris with only Dsg 3 antibodies has only mucosal involvement whereas patients with both Dsg1 and Dsg3 antibodies develop both cutaneous and mucosal lesions(26)(32)(33).The desmosomal compensation theory is depicted in figure 4.



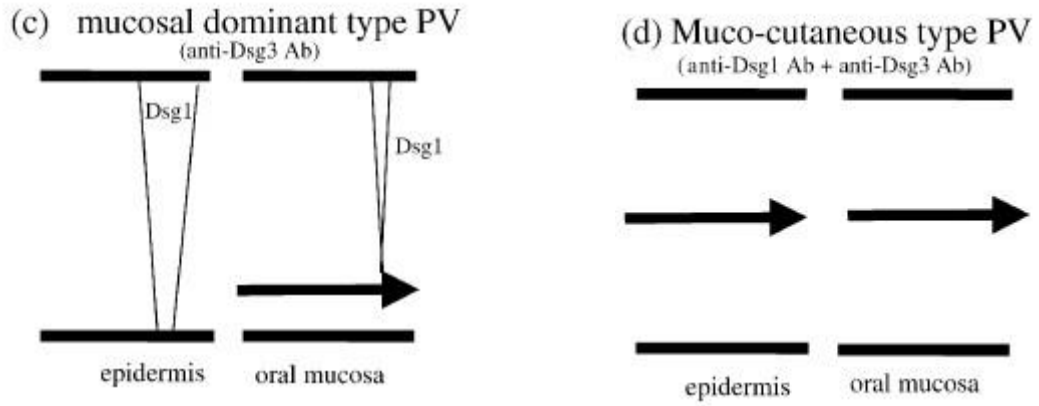


Figure 4 Logical explanation of localization of blister in pemphigus by desmoglein compensation theory(27).

Stanley et al. concluded that anti Dsg1 and Dsg3 antibodies are sufficient enough to induce blisters in pemphigus vulgaris patients. But in occasional cases it is observed that there is inconsistensies between desmoglein titres and the clinical phenotype of pemphigus.

Following are the inconsistencies observed and questions raised regarding the pathogenicity of desmoglein antibodies in pemphigus vulgaris patients.

1) Desmoglein1 and Dsg3 antibodies present in pemphigus vulgaris patients causes suprabasal rather than subcorneal and suprabasal blisters.

2) Some individual harbor both Dsg1 and Dsg3 antibodies but do not manifest pemphigus.

3) Kricheli et al. observed the presence of anti desmoglein antibodies in disease free relatives of pemphigus patients and only in 62% of pemphigus vulgaris patients(34).

4) Sera of patients with periodontitis and silicosis developed both anti Dsg1 and anti Dsg3 antibodies(35).

The following observations suggest that nondesmoglein antigens may be responsible for blistering in pemphigus vulgaris:

1. High titres of desmoglein 3 antibodies were found to be present in the first degree relatives of pemphigus patients but apparently failed to produce blisters unlike in pemphigus patients(36).

2.Pemphigus vulgaris autoantibody IgG representing extracellular domain of Dsg3, when injected into neonatal mice showed signs of cell-cell detachment visible on microscopy failed to produce gross skin blistering even though appreciable titre of antibody was present in mouse serum(37).

3.Cholinergic agonists reversed acantholysis both in vivo and in vitro(38).

There has been always a correlation between disease severity and desmoglein antibody titres, but however in some cases of pemphigus, it doesn't strictly correlate. Furthermore, anti desmoglein antibodies can be absent during the active stage of disease but can be present during remission, in patients with other medical conditions and also in healthy people. The above data suggests that desmoglein antibodies and nondesmoglein antibodies together mediate the pathogenicity of pemphigus vulgaris. Nguyen et al. concludes that the missing component in the pathogenesis is the antibodies to cholinergic receptors on the surface of the keratinocytes(39).

<u>Antibodies other than desmoglein postulated in pemphigus:</u>

Nguyen et al. reported that pemphigus vulgaris autoantibodies with anti Dsg 3 activity and absent Dsg1 activity can induce blisters in Dsg 3 deficient mice(40). This observation

led to detection of multiple non desmoglein antigens by probing immunoblots of epidermal extracts using pemphigus vulgaris sera. Grando and colleagues proposed unifying hypothesis which states that blistering in pemphigus vulgaris is the result of synergy between anti-cholinergic receptor antibodies and anti-desmoglein antibodies(41). Facts in favour for pathogenic roles for nondesmoglein antibodies to keratinocyte receptor

1) Nguyen et al. detected cholinergic receptor antibodies by radioimmunoprecipitation assay in 85% of pemphigus vulgaris and pemphigus foliaceous patients(42).

2) Nguyen et al. observed acantholysis in keratinocyte monolayers of human skin induced by anti-α9 antibody(43).

3) Nguyen et al. observed shedding of desmosomes by anti pemphaxin antibody and its adsorption prevented acantholysis by pemphigus vulgaris IgG. He also postulates that preabsorbed serum restores its acantholytic activity after addition of anti pemphaxin antibody(44).

4) Grando reviewed the pathogenesis of pemphigus and concludes that cholinergic receptor antogonists causes acantholysis in keratinocyte monolayers whereas cholinomimetics restores acantholysis and counteracts pemphigus vulgaris IgG acantholytic activity in vitro and hence cholinomimetics are used in the treatment of pemphigus in vivo(45).

5) Hu et al. observed that abnormal intracellular calcium concentration resulted in acantholysis in Hailey Hailey disease which is due to mutation in the gene ATP2C1. Sakunthabai et al. observed acantholysis in Dariers disease due to abnormal intracellular

calcium concentration as a result of mutation in the gene SERCA type2, ATP2A2(46)(47).

By radioimmunoprecipitation assay using acetylcholine receptor derived from epidermal keratinocyte and covalently labeled with muscarinic radioligand as an antigen, Nguyen et al. detected that 85% of pemphigus patients have anti AChR antibody(43). He demonstrated that rDsg3-Ig-His adsorbs both desmoglein and nondesmoglein autoantibodies to various keratinocyte antigens. He also identified acetylcholine receptor antibodies targeted receptors by preincubating monkey esophagus with pemphigus vulgaris antibodies. Antibody to α9 acetylcholine receptor with both muscarinic and nicotinic actions stained keratinocytes in a fishnet pattern. Alpha 9 acetylcholine receptor antibodies induced pemphigus like acantholysis in keratinocyte monolayers. Acantholysis was reversed spontaneously or by cholinergic agonist carbechol.

Acetylcholine receptors on keratinocytes:

Epidermis has both muscarinic and nicotinic receptors(32)(48)(49). There are 5 types of muscarinic receptors M1, M2, M3, M4, M5 and 7 types of nicotinic receptors α3, α5, α6, α7, β1, β2, β4 in the epidermis. M3 is predominantly situated in suprabasal layer, M4 and M5 in the prickle cell layer and M1 in superficial layer. Nicotinic receptors are present in suprabasal layer. Alpha9 acetylcholine receptor and pemphaxin are the recently described acetylcholine receptors in the pathogenesis of pemphigus. Alpha 9 acetylcholine receptor is of both muscarinic and nicotinic type. Pemphaxin is a protein present on the keratinocytes and acts as cholinergic receptors by binding with acetylcholine. Pemphaxin is a homodimer with 40kDa subunits. All these acetylcholine

receptors helps in maintaining the shape and adhesion of keratinocytes. Muscarinic receptors exerts its action by releasing intracellular stores of calcium whereas nicotinic receptors does it by increase in the influx of calcium into the cell.

Keratinocytes synthesize and release endogenous acetylcholine in a autocrine and paracrine manner. This endogenously produced acetylcholine constantly activates acetylcholine receptors to maintain the adhesive function of keratinocytes. Similarly both muscarinic and nicotinic agonists maintains the adhesive function of keratinocytes. Muscarinic or nicotinic antagonists like atropine and mecylamine respectively blocks acetylcholine receptors in the keratinocyte monolayers resulting in pemphigus like acantholysis. Cholinergic agonists of both classes reverses acantholysis as well as protects keratinocyte monolayers from the acantholytic effects of pemphigus antibody. Thus all the above observations suggests that keratinocyte adhesion and motility controlled by endogenous acetylcholine is antagonized by pemphigus autoantibody by acting on the acetylcholine receptors on the surface of keratinocytes resulting in acantholysis.

The mechanism by which acetylcholine receptor antibodies resulting in acantholysis is depicted below as schematic diagram.(fig.5)

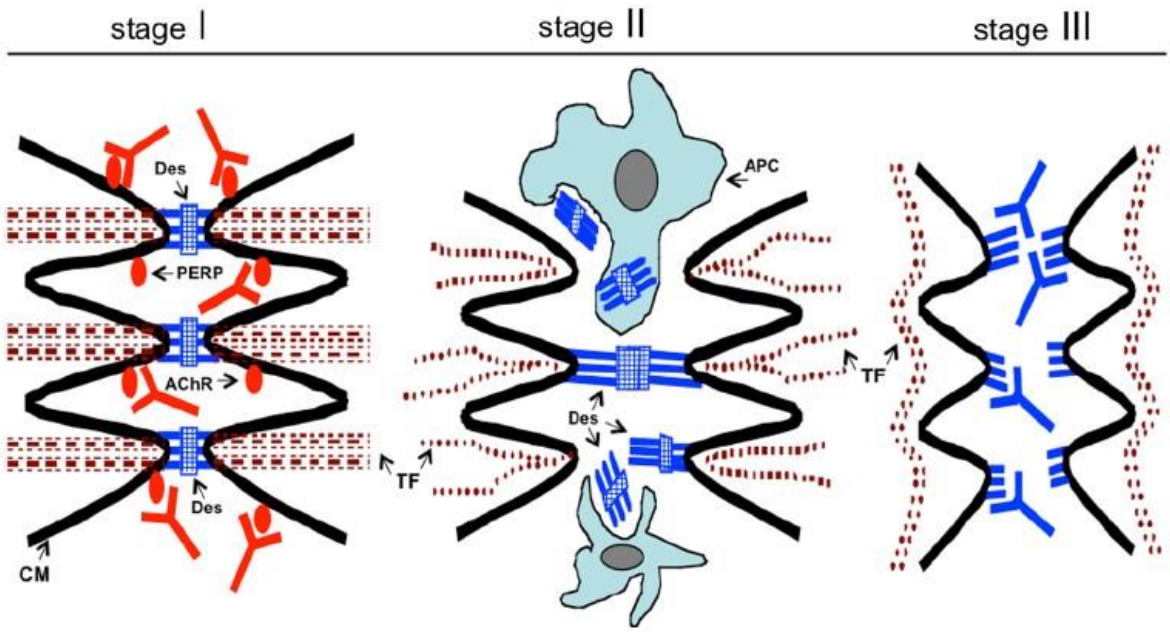


Figure 5 Hypothetical scheme of the time course of pathobiologic events leading to acantholysis in pemphigus(32).

1) In stage 1, anti acetylcholine receptor antibodies binds to its receptors on the keratinocytes and interferes with the physiologic control of intercellular adhesion and its polygonal shape. There is also initiation of programmed cell death and increased phosphorylation of adhesion molecules resulting in its dissociation.

2) In stage 2, there is shrinkage of keratinocyte due to collapse of tonofilament resulting in sloughing of desmosomes thereby stimulating secondary antibody production against desmosomal antigens.

3) In stage 3, anti desmoglein antibodies binds to keratinocytes and prevents the formation of new desmosomes.

Grando hypothesized that several acetylcholine receptor autoantigens are involved in the pathogenesis of pemphigus patients. Grando detected two cholinergic receptors in most

of the pemphigus vulgaris patients. The two receptors involved in the pathogenesis of pemphigus patients were α9 acetylcholine receptor and pemphaxin which is a novel keratinocyte annexin like molecule that binds to acetylcholine(40).

In neonatal mice, the pathogenicity of pemphigus sera is depleted by preabsorbing the pemphigus sera with pemphaxin. Antibody directed against pemphaxin caused acantholysis in keratinocyte culture. However it is noted that the eluted material is not pathogenic and hence suggested that the antibody against pemphaxin is necessary but not sufficient to be pathogenic. The effect of corticosteroids on acantholysis in pemphigus vulgaris patients is by boosting the pemphaxin expression.

Nguyen et al. reported a case of pemphigus vulgaris who improved with cigarette smoking thereby paving the way for the use of nicotinamide as steroid sparing agent(43).

Grando postulated multiple hit hypothesis which states that acantholysis in pemphigus is due to antibodies against different keratinocyte antigens which includes regulatory molecule such as AChR and structural molecule such as desmosomal cadherin(12).

There is still a debate that whether cholinergic receptor antibodies represent an epiphenomenon secondary to acantholysis or is it a pathogenic antibody involved in pemphigus patients. However identification of antigens targeted by PV autoantibodies is still under intense research.

Clinical features:

Pemphigus vulgaris is clinically divided into 3 subgroups 1) Mucosal dominant type with mucosal lesions and minimal skin lesions 2) Mucocutaneous type with extensive cutaneous lesions with mucosal lesions 3) Cutaneous type PV without apparent mucosal involvement is observed as a rare clinical and histologic expression of pemphigus. This expression can be a transient phenotype that may develop from, or evolve into, other subtypes of pemphigus(50). In majority of pemphigus vulgaris patients, oral mucosal involvement is the initial manifestation(13). Oral lesions either precedes cutaneous lesions or may be the only manifestation. About 50% - 70% of pemphigus vulgaris patients presents with oral lesions. They present with non-healing painful ulcerations in the mouth(13). The most commonest site of involvement are buccal mucosa and palate followed by labial mucosa and tongue(13). Oral erosions are usually multiple and of variable sizes with an irregular, ill-defined border(51). Extensive oral mucosal involvement results in severe pain and decreased oral intake. The diagnosis of pemphigus is considered in patients presenting with non-healing oral ulcers persisting for more than a month(13). Other mucosa which can be affected are nasal mucosa, conjunctiva, oesophagus, pharynx, larynx, anal mucosa, urethral mucosa, vagina, labia, cervix and penile mucosa. Involvement of throat is associated with difficulty in swallowing.

Pemphigus vulgaris patients develop cutaneous lesions after few weeks to few months whereas there are occasional reports of some patients presenting with only cutaneous lesions without mucosal lesions. Predominant cutaneous sites of involvement are scalp. face, axilla, groin and pressure points. Usually upper torso and central portion of the body

is commonly involved. Cutaneous lesions comprises of clear fluid containing flaccid blisters on a normal or erythematous skin. These clear flaccid blisters later becomes haemorrhagic or turbid and spontaneously ruptures to form painful erosions which has the tendency to extend. These erosions later on becomes crusted, oozes and bleeds. If left untreated cutaneous erosions extends and becomes widespread resulting in complications like secondary infection or metabolic disturbances or both leading to death. Though pruritus is not a common symptom of pemphigus patients, its presence indicates that disease is active and predicts relapse. Skin lesions usually heals with hyperpigmentation or acanthomata but without scarring. Acral involvement is rare but its presence denotes poor prognostic indicator of the disease(17). Unusual clinical manifestations of pemphigus vulgaris are 1) isolated crusted plaque on face or scalp 2) paronychia 3) foot ulcers 4) dyshydrotic eczema or pompholyx and 5) macroglossia(51). There are certain clinical signs which are elicited bed side in pemphigus vulgaris patients and these signs are helpful to differentiate between intraepidermal and sub-epidermal bullous disorders.

Nikolskiy sign:

When a tangential pressure is applied with a finger pad or thumb to the margin of an erosion or bulla, it forms a erosion due to cleavage of upper layer of epidermis from lower layer of epidermis(31).This is known as marginal nikolskiy sign and is positive in pemphigus. If the same is elicited over a normal looking skin away from the erosion or bulla, then it is named as direct or distant nikolskiy sign. This is the first sign to disappear with treatment whereas its presence indicates disease severity.

The nikolskiy phenomenon:

It is the formation of bulla after some time instead of erosion when similar kind of tangential pressure is applied.

The modified nikolskiy sign:

It is the peripheral extension of bulla when vertical pressure is applied its surface and it is useful when no new bulla is available for biopsy. The newly formed bulla does not show re-epithelisation which is seen in older subepidermal bulla thus making it to appear intraepidermal.

Bulla spread sign:

It is also known as Lutz sign. It is elicited by first marking the margin of the bulla by pen and then exerting slow unilateral pressure by a finger which extends the bulla margin beyond the marked margin. In pemphigus the newly formed bulla has irregular and angulated border whereas it is uniform and rounded in subepiderma bulla.

Asboe Hansen sign:

It is applied if the bulla is smaller and tense bulla, pressure is applied vertically on the surface of the bulla.It is positive in all cases of pemphigus.

DIAGNOSIS:

Diagnosis of pemphigus is based on clinical features, histopathology, DIF and indirect immunofluorescence study.

Tzanck test:

This test is very useful in early lesions of oral pemphigus. It is a simple, cost effective, rapid bedside test to diagnose pemphigus. With the help of blade the roof of newly formed bulla is deroofed and fluid present in the bullous cavity is completely drained off and the base is scraped and made into a smear on a clean glass slide and stained with leishman stain. Slide is then viewed under microscope under oil immersion for the presence of multiple acantholytic cells. An acantholytic cell is a large round keratinocyte with hyperchromatic nucleus, absent nucleolus and peripheral rim of basophilic cytoplasm with a perinuclear halo(52)(53)(54). Two types of acantholytic cell was described by Desai and Rao. Type A cells has large noncondensed nucleus, fuzzy basophilic cytoplasm and a perinuclear halo whereas type B cells has condensed pyknotic nucleus and well defined eosinophilic cytoplasm(17). Depicted below is a picture of acantholytic cells. (fig. 6)

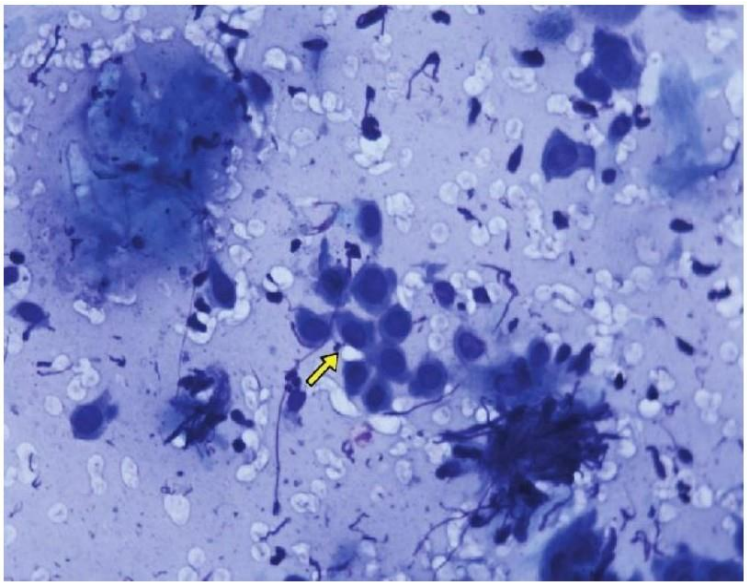


Figure 6 Tzanck smear showing acantholytic cells(31).

Cell adherence is less characteristic sign in pemphigus vulgaris. “Sertoli rosette” is where a ring of lymphocytes surrounds a central epithelial cell. “Streptocytes” is formed by a chain of lymphocytes which adhere together by glue like material(52).

Histopathology:

Skin biopsy is done from a fresh vesicle. Early histopathologic changes are eosinophilic spongiosis followed by separation of suprabasal keratinocytes from the basal cells forming a suprabasal bulla which contains acantholytic cells. Basal cells lose their adherence with the neighbouring cells but remain attached to basement membrane thus appearing as “row of tomb stones”. Upper dermis contains mixed inflammatory infiltrate. The picture of histopathology of pemphigus vulgaris is given below.(fig.7)

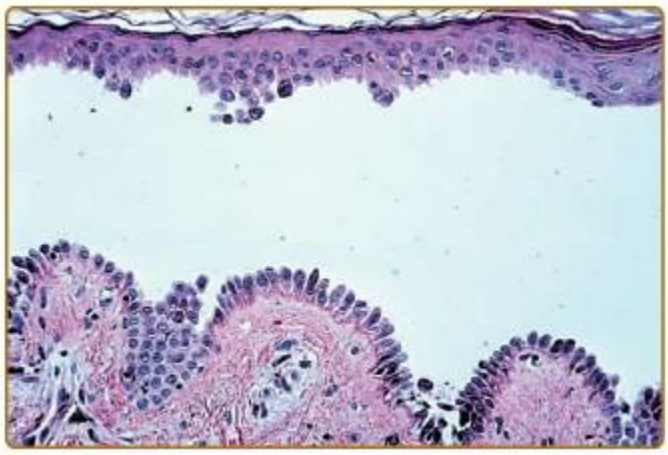


Figure 7 Histopathology of pemphigus showing suprabasal bulla and row of tomb stone appearance(55).

Electron microscopy:

There is widening of intercellular space followed by splitting of desmosomes resulting in separation of keratinocytes. Tonofilaments were found to retract and clump around the nucleus and also there is disappearance of desmosomal plaques.

Immunoflourescence staining:

All pemphigus vulgaris patients have both skin fixed and circulating antibodies against keratinocyte surface antigens. About 90% of pemphigus patients have skin fixed intercellular antibodies are in both lesional and nonlesional adjacent healthy skin. The presence of intercellular tissue fixed antibodies are detected by DIF (direct immunofluroscence) and circulating antibodies in the serum is detected by IIF (indirect immunofluroscence)

Direct immunofluorescence:

DIF of peri-lesional skin:

It is a reliable diagnostic tool for pemphigus vulgaris. It is a nonquantitative test. All pemphigus vulgaris patients have positive DIF of perilesional skin. This test helps to detect the presence of IgG and C3 bound to cell surface of keratinocytes in the perilesional skin because immunoreactants are absent in the lesional skin. IgG is deposited in the intercellular junctions of epidermal keratinocytes and thus gives a fishnet appearance in DIF of pemphigus vulgaris patients. Increased complement levels or its re-appearance indicated impending relapse(17). The picture of direct immunofluorescence of skin is given below.(Fig. 8)In some patients, DIF may remain positive for many years even after clinical remission.

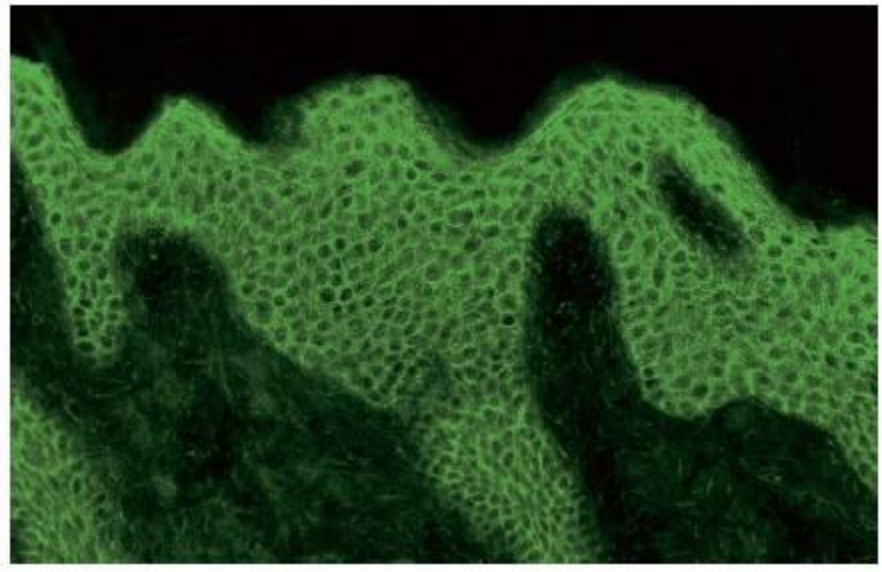


Figure 8 Direct immunofluroscence study of pemphigus vulgaris patient showing fish net pattern of deposition of intercellular IgG antibodies(15).

DIF of outer root sheath:

Studies shows that DIF of outer root sheath of plucked hair can be used as tool for the diagnosis of pemphigus vulgaris. Epidermis continues as outer root sheath and so if DIF is positive in epidermis then it will also be present in the outer root sheath of plucked hair. In a Indian study of pemphigus vulgaris patients, it was found that 85% had DIF positivity of plucked hair. Thus it is a simple, non-invasive and specific test which can be used to monitor disease activity(17).

Indirect immunoflouroscence:

Indirect immunofluorescence assays detects circulating intercellular antibodies present in the patients serum using a substrate like monkey oesophagus, guinea pig oesophagus or human skin. Majority (80%) of pemphigus patients in active disease have circulating intercellular antibodies in their serum(55). If indirect immunofluorescence is negative in pemphigus patient, then test has to be repeated with another substrate. Certain amount of circulating antibodies are detected by indirect immunofluroscence in patients with fungal infections, burns, myasthenia gravis, drug reactions and inpatients with antibodies to ABO blood groups(15). Disadvantage of indirect immunofluroscence is that it does not helps to differentiate between pemphigus vulgaris and foliaceous(55). It remains positive for a long time after clinical remission even in patients without active lesions. It is a useful guide for monitoring therapy. Its titre correlated well with disease activity except in certain cases. Indirect immunofluroscence performed with blister fluid showed similar results like IIF performed with serum. It is a non-traumatic and simple procedure useful in the diagnosis of pemphigus vulgaris(17).

ELISA assay of recombinant Dsg1and Dsg3:

Enzyme –linked immunosorbent assay of recombinant Dsg1 and Dsg3 detects circulating IgG antibodies in the serum of pemphigus vulgaris patients. It is more sensitive and specific test used for the diagnosis of pemphigus vulgaris patients. It is a quantitative analysis of antibodies level whereas IIF is a semiquantitative test(9). There is increased levels of Dsg3 in pemphigus vulgaris patients with predominant mucosal lesions whereas Dsg1 is raised up in PV patients with mucocutaneous lesions. In an Indian study on 44 pemphigus patients, it was concluded that desmoglein antibodies levels correlated well with disease severity. However a small proportion of cases did not correlate because of the presence of pathogenic antibodies to nondesmoglein molecules or intracellular domain of Dsg1 and Dsg3 which is undetected by Dsg1 and Dsg3 specific ELISA(9). In case of high antibody concentrations, the assay plates become saturated and so ELISA results are not quantitative.

In a study by of 35 pemphigus patients done by Patsatsi et al. disease activity was measured by means of PDAI and ABSIS disease activity scoring and concurrently correlated with Dsg1 and Dsg3 antibodies levels. This study showed that Dsg1 antibodies titre correlated well with disease activity but Dsg3 antibodies titre did not correlate(56). About 27 pemphigus patients was included in an Indian study by Sharma et al. and disease activity was correlated with desmoglein 1 and 3 antibodies titre. It was concluded that desmoglein antibody titre correlated well with disease activity but its titres failed to correlate with its morphologic subtypes of pemphigus vulgaris(57).

Recently, new immunologic targets involved in pathogenesis of pemphigus vulgaris have been discovered which includes acetylcholine receptor. Various studies have been conducted to determine the relationship of development of pemphigus vulgaris and acetylcholine receptor antibodies. In one such study by Sanchez et al. in which he included 31 pemphigus patients and measured disease severity by BSA and correlated with desmoglein and acetylcholine receptor antibodies at the time of diagnosis and in the follow up. This study concluded that acetylcholine receptor antibodies correlated with disease severity at the time of diagnosis and follow up. But it is still not clear whether it is a potential trigger of pemphigus or just an epiphenomenon(11).

Scoring systems in pemphigus:

As patients with pemphigus present with various protean manifestations, there are various therapeutic options available with variable outcome. This led to the need for clinical scoring system to monitor the activity and its response to treatment.

Pemphigus disease activity index:

Pemphigus disease area index scoring was developed by international pemphigus committee in 2007(8)(58). It assess both cutaneous and mucosal lesions, its number and sizes and also postinflammatory hyperpigmentation of resolving lesions(59). Many studies were conducted to validate scoring system. In one such study, PDAI scoring was compared to ABSIS and it was concluded that PDAI is quick, easy and reliable method to score both cutaneous and mucosal lesions(59)(8). Assessment of type of lesions, Nikolskiy sign or use of rule of nine is not required in PDAI scoring thereby eliminating the source of variability(8). The possible score of PDAI ranges from 0 to 263. Out of 263

points, 250 points denotes disease activity (120) points each for cutaneous lesions and mucosal lesions and 10 points for scalp lesions). Thirteen points was allotted to disease damage(58).

Autoimmune bullous skin disorder intensity (ABSIS):

It is a quantity and quality based scoring for both mucosal and cutaneous lesions(59). It is also used for other immunobullous disorder.

Saraswats oral pemphigus scoring:

It scores only mucosal lesions(59).

Various other scoring system used for pemphigus includes Mahajan's scoring system, Kumar's scoring system and Harman's pemphigus grading.

Treatment:

The main stay of treatment for pemphigus vulgaris is systemic corticotseroids. After the advent of steroids the mortality rate of pemphigus is reduced from 90% to 33%(60).

Treatment of pemphigus is divided into 3 phases 1) control phase 2) consolidation phase 3) maintenance phase(13).The main aim of treatment is bring the disease under control as early as possible.

Control phase:

In this phase disease is brought under control by rapidly increasing the intensity of treatment until reduction or complete suppression of new lesion occurs, established lesions begins to heal. Pemphigus usually responds within 2 weeks. Initial dose of steroid

is 0.5mg to 1.5mg/kg/day(61). If the disease is not under control, then the dose of steroid is increased by 50% every 1-2 weeks until disease is controlled(13).

Consolidation phase:

In this phase the dose of steroid needed to control the disease is maintained until 80% of established lesions have healed(13).

Maintenance phase:

This phase begins when most of the established lesions have resolved. In this phase the doses of steroid is tapered gradually to lowest dose to suppress the occurrence of new lesions(13). As higher and prolonged doses of steroid can have deleterious side effects, adjuvant drugs which have steroid sparing effect is added to the treatment. Some of the adjuvant drugs commonly used are

1. Azathioprine in the dose of 1-3mg/kg/day. The levels of TPMT (thiopurine methyl transferase activity) is measured before initiating treatment because the dose of azathioprine depends on it. If the TPMT activity is high, then patient is treated with normal doses of azathioprine 2.5mg/kg/day. If TPMT activity is low, then the dose of azathioprine is adjusted to low dose of 0.5mg – 1.5mg / kg / day. Absence of TPMT activity is an indication for avoidance of azathioprine(61).
2. Mycophenolate mofetil 2g/ day. The dose of MMF is gradually increased by 500mg every week until 2g/day is reached.
3. Cyclophosphamide 2mg/kg/day or as DCP pulse (dexamethasone, cyclophosphamide pulse therapy)
4. Methotrexate 10-20mg/week

5. Dapsone 100mg / day

Other modalities of treatment include Intravenous immunoglobulin (IVIgG) and plasmapheresis. Localised lesions are treated with intralesional and topical steroids. Oral lesions are treated with topical steroid. In case of refractory cases biological treatment with humanized antiCD20 monoclonal antibody known as rituximab results in complete healing of all lesions(62).

According to multiple hit theory, cumulative effects of nondesmoglein target antigen like acetylcholine receptor is necessary for the process of acantholysis(45). Studies also proved that titres of acetylcholine receptor antibodies correlated with disease activity and desmoglein titres. This led to the introduction of cholinomimetic drugs for the treatment of pemphigus vulgaris patients(11). To ameliorate the side effects of steroid, newer non-hormonal treatment of pemphigus with cholinergic agonists was suggested. Grando, in his clinical trial on mestinon (pyridostigmine) in pemphigus patients demonstrated that mestinon can be used for mild disease with chronic lesions on limited areas(63). Nguyen et al. in his study concluded that pyridostigmine bromide (acetylcholine esterase inhibitor) helped to maintain steroid at a lower dose in pemphigus patients than before initiating pyridostigmine treatment(64).

MATERIALS AND METHODOLOGY

Study design:

It is a study of acetylcholine receptor antibodies done to correlate with disease activity measured by PDAI and to compare with desmoglein titres in pemphigus vulgaris patients.

Setting:

The study was conducted in the Outpatient Department of Dermatology, Venereology and Leprosy, Christian Medical College and Department of Biochemistry in a tertiary care hospital in South India.

Duration of the study:

The study was conducted between period of October 2014 and August 2015(13 months).

Study population:

Inclusion criteria:

All patients diagnosed to have pemphigus vulgaris based on clinical (presence of mucosal and/ or cutaneous erosions) and histological (intraepidermal or suprabasal acantholysis) or DIF (deposition of IgG and/or C3 between keratinocytes) and who consented to participate were included in the study.

Exclusion criteria:

Patients with Myasthenia gravis, Sjogrens syndrome and Thymoma were excluded from our study.

Methodology:

All patients who conformed to the inclusion criteria were examined by the principal investigator after a written consent was obtained.(Annexure 1) Details on demography, duration of the disease, site of onset of lesion, duration and details of treatment taken were recorded in a proforma.(Annexure 2) Patients were categorised according to the clinical variants of pemphigus (pemphigus vulgaris, pemphigus foliaceous and pemphigus vegetans). Patients with PV were further categorised into variants as:- mucocutaneous, mucosal dominant or cutaneous dominant pemphigus. They were assessed at the time of their presentation for clinical severity of their disease with the help of PDAI clinical scoring.(Annexure 2)

The PDAI score was calculated by assessing the number and the size of the lesions present on the skin, scalp and mucosa. The disease activity on the skin was calculated as the sum of the scores on 12 predetermined areas. The score on each area was graded as: 0, 1, 2, 3, 5 and 10. The disease activity on the scalp was determined by the number of quadrants involved and was graded as 0, 1, 2, 3, 4 and 10. The activity on the mucosa was scored as: 0, 1, 2, 5 and 10 based on the number and size of the erosions on 12 sites. The damage score in each area was taken as 1 if resolving erythema/ hyperpigmentation was present and 0 if absent.

Desmoglein1 and desmoglein3 antibody titre was evaluated by ELISA method for all the patients. Desmoglein estimation is done by M.B.L.Co.Lt desmoglein ELISA kit (Japan).

Readings more than 20u/ml is taken as positive titre. In addition to this, estimation of AChRAb titre was done by RSR's AChRAb ELISA kit (RSR Limite78, Avenue Park Pentwyn Cardiff CF23 8HE, United Kingdom). The value AChRAb titre was correlated with disease severity and then compared with desmoglein antibody titre.

Estimation of AChRAb titre:

About 5ml of blood was collected in a vaccutainer test tube from the patients and transported immediately to biochemistry department for centrifugation and sera was stored at -20˚c in aliquots for analysis. At the time of analysis sera was removed from the refrigeration and thawed.

Assay Principle:

RSR's AChRAb ELISA depends on the ability of AChRAb in human serum to bind to similar sites on the AChR as various monoclonal antibodies such as MAB1(coated on ELISA plate wells) and MAb2 and or MAb3(which are labeled with biotin).In the absence of AChRAb a complex is formed between MAb1 coated on the plate wells, the AChR and MAb2- and MAb3-Biotin. MAb2- and MAb3- Biotin bound are then detected by addition of streptavidin peroxidase (SA-POD), which binds specifically to Biotin. Excess, unbound SA-POD is the washed away and addition of the peroxidase substrate 3,3',5,5' – tetramethylbenzidine (TMB) results in formation of blue colour. This reaction is stopped by addition of stop solution causing the well contents to turn yellow. The absorbance of the yellow reaction mixture at 450nm is then read using an ELISA plate reader. In the presence of AChRAb the formation of the MAb1-AChR-MAB2 biotin

complex is inhibited, resulting in less SA-POD being bound and a reduction in final absorbance at 450nm. The higher the concentration of AChRAb in the test serum, the greater the inhibition of ACRAb in the test serum, the MAb-Biotin binding. The absorbance of each well is read at 450nm using an ELISA plate reader..

Result analysis:

A calibration curve is drawn by plotting calibration concentration on the x-axis against the absorbance of the calibrators on the y-axis. The AChRAb concentrations in patients sera is then read off the calibration curve. The assay cut off value is < 0.45nmol/L for negative and ≥ 0.45nmol/L for positive patients.

Sample size:

A sample size of 12 patients are needed to detect a correlation of 0.78 among disease extent and acetylcholine receptor antibody level with 80% power and 5% error.

Following formula was used to calculate sample size.

$$n = \frac{\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}\right)^2}{[FZ(\rho_1) - FZ(\rho_0)]^2} + 3$$

$$FZ(\rho_1) = \frac{1}{2}\ln\left[\frac{1+\rho_1}{1-\rho_1}\right] \qquad FZ(\rho_0) = \frac{1}{2}\ln\left[\frac{1+\rho_0}{1-\rho_0}\right]$$

Where,

ρ_0 : Population correlation coefficient

ρ_1 : Sample correlation coefficient

$Z_{1-\alpha/2}$: Desired confidence level

$1-\beta$: Power

Statistical analysis:

The statistical analyses were performed using Windows Office Excel 2007 and SPSS 12.00 for Windows. The continuous variables were summarised as mean with standard deviation or median with maximum and minimum values of the ranges. The categorical variables were summarised as numbers and percentages. The relationships between continuous variables were assessed using Pearson's product moment correlation with scatter plots as diagrammatic representations of the correlation.

Institutional review board:

The study was approved by the Institutional review board.

RESULTS

A total of 77 patients with pemphigus, who met the inclusion criteria were enrolled into the study during the study period of October 2014 to August 2015.

Demographic details:

Age distribution: The mean age of the study population was 46 years +/- 13years. The youngest patient was 22years old and the oldest patient age was 79years old. Maximum number of patients (70%) were more than 40yrs of age. This is depicted in figure 9.

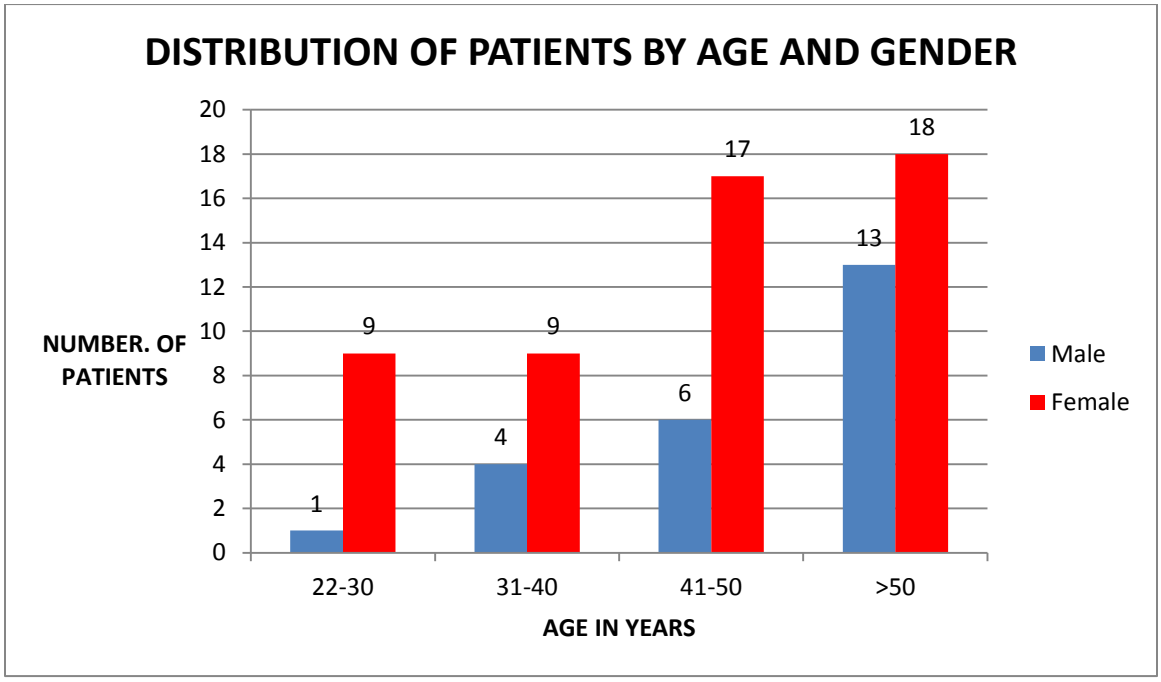


Figure 9 Distribution of patients by age and gender.

Females: Females outnumbered males in all the age groups. There was an equal distribution of female participants (34%) in the age group of 22 - 30yrs and 31 – 40 yrs. Majority of female participants (66%) belonged to the age group of 41- 50yrs and above 50 yrs.

Males: Majority of male patients 54.2% were more than 50 yrs old. There was only one patient in 22 – 30 yrs age group.

Gender distribution of the patients:

There were totally 53 females (68.83%) and 24 males (31.17%). The female to male ratio was 2.2:1. This is depicted in figure 10.

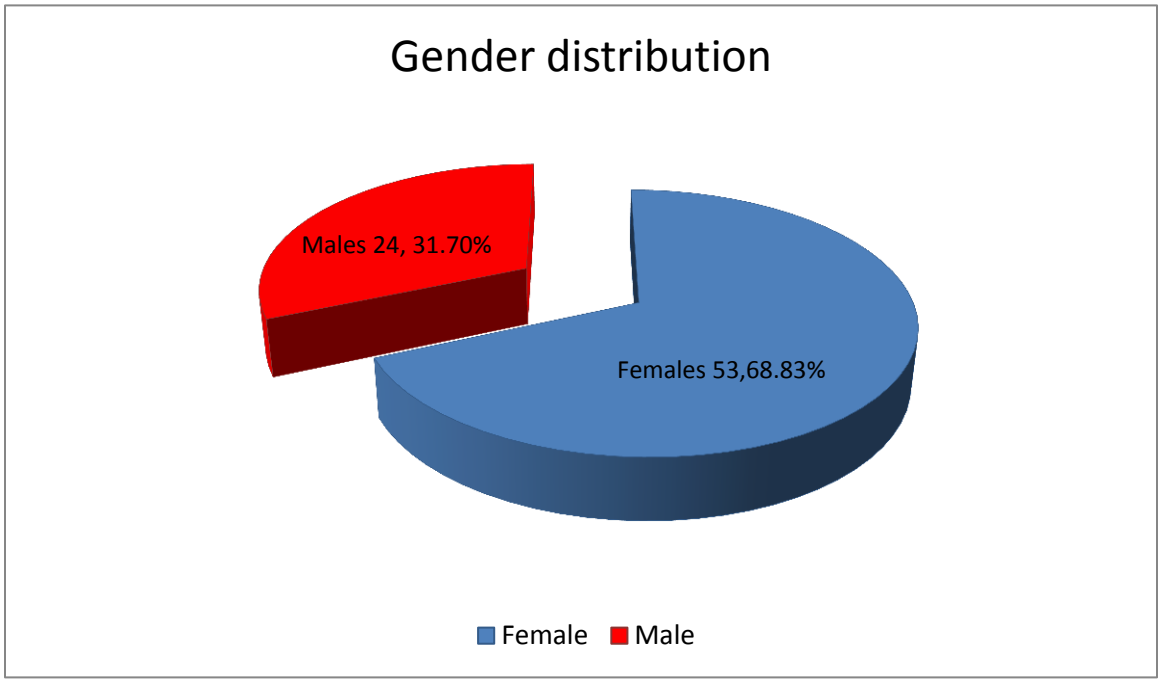


Figure 10 Gender distribution in the study population.

Duration of lesions:

The duration of skin lesions ranged between 2-120 months and of that of oral lesions ranged from 2 – 210 months. The mean duration of skin lesions was 30.35 +/- 35.95 months and median duration was 12 months. The mean duration of oral lesions was 32.9 +/- 36.79 months and median duration was 24 months.

Onset of lesions:

At presentation 24 of 77 patients (31.2%) had both cutaneous and oral lesions, 19 had only cutaneous lesions (24.7%) and 15 had only oral lesions (19.5%). The most common site of onset of cutaneous lesion was chest. Similarly, the most common site of onset of oral mucosal lesion was buccal mucosa.

Clinical subtypes of pemphigus vulgaris:

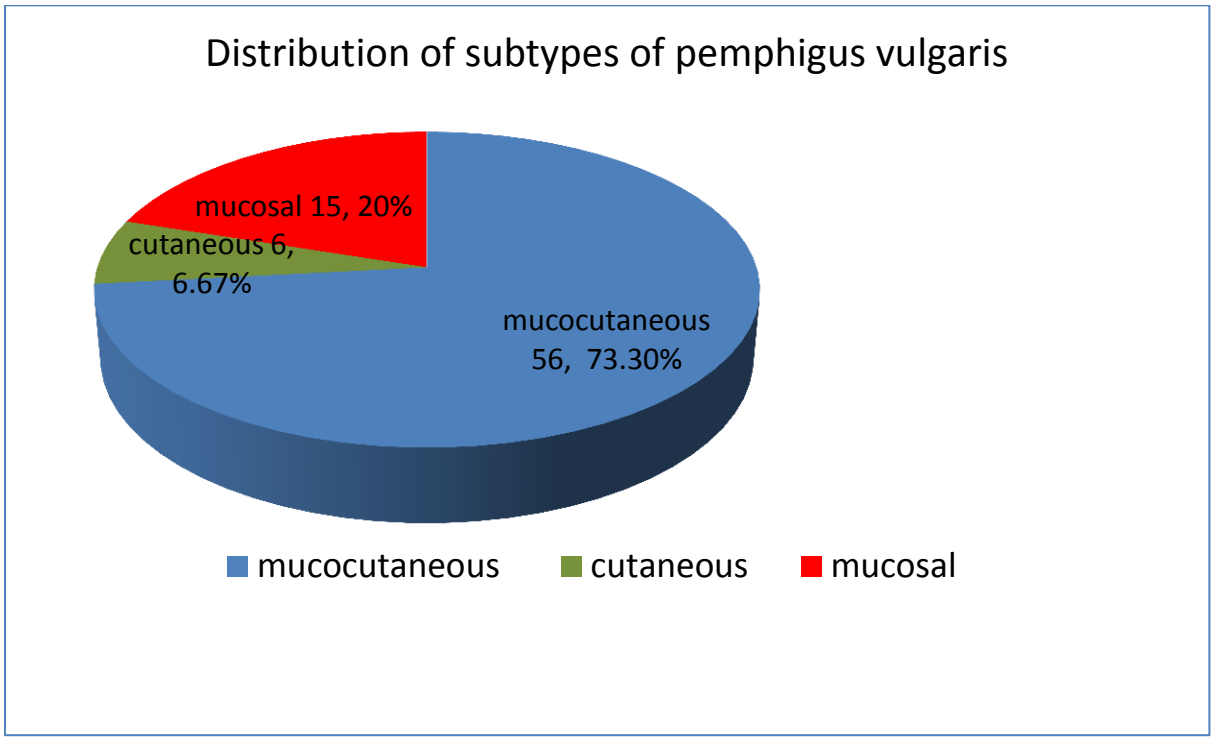


Figure 11 - Distribution of clinical subtypes of pemphigus vulgaris in the study population.

The figure 11 shows the distribution of the subtypes of pemphigus vulgaris in the study population. Majority (73%) of patients with PV had mucocutaneous involvement. About 20% of the population had purely mucosal and 6% had predominantly cutaneous disease.

Distribution of oral mucosal lesions:

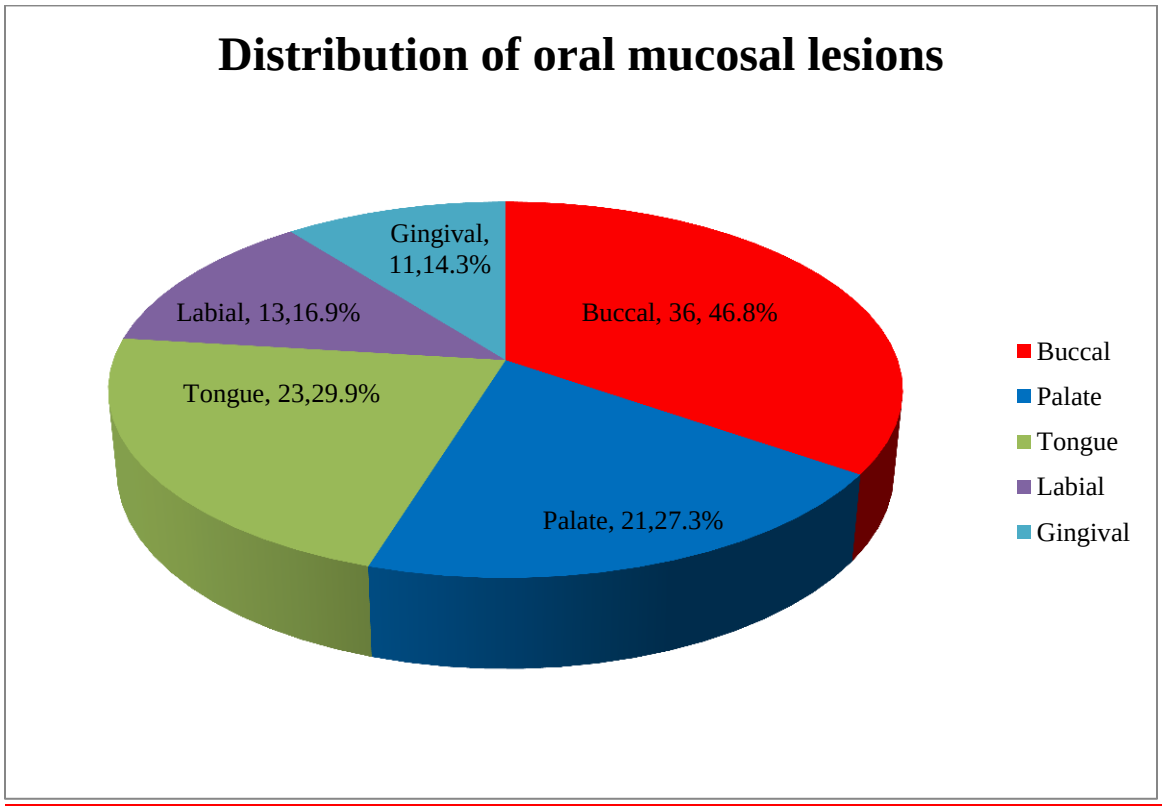
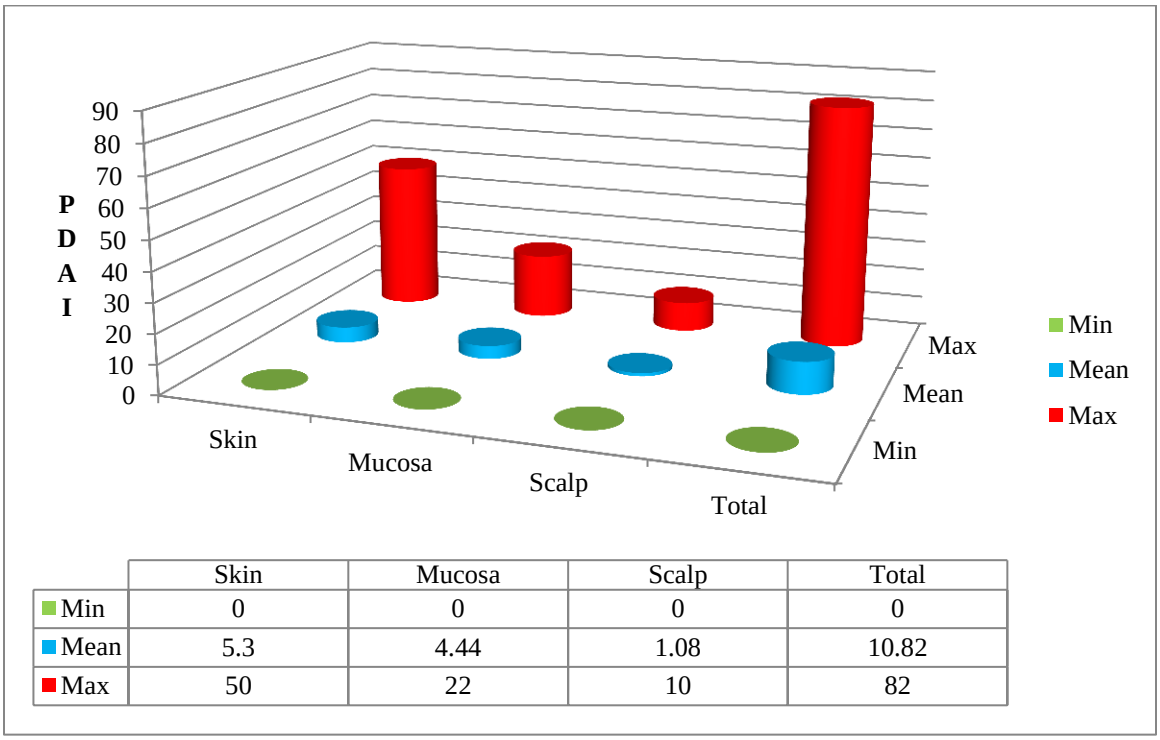


Figure 12 - The piechart shows the distribution of oral mucosal lesions in both mucosal and mucocutaneous subtype of pemphigus patients.

The chart (fig.12) shows majority of patients (46.8%) had buccal involvement followed by tongue, palate, labial and gingiva. Other mucosa involved were nasal and genital mucosa in 3 patients each.

PDAI clinical scoring for disease activity:



	Skin	Mucosa	Scalp	Total
Min	0	0	0	0
Mean	5.3	4.44	1.08	10.82
Max	50	22	10	82

Figure 13 - PDAI clinical severity scores of skin, mucosa, scalp and total score

The graph (fig.13) shows the minimum, maximum and mean value of PDAI score of skin, mucosa, and scalp.

- The minimum score is zero for all the sites.
- The maximum score for total skin was 50 out of 132 and the mean was 5.3 +/- 8.6.
- The maximum score for total scalp was 10 out of 11 and the mean was 1.08 +/- 2.08.
- The maximum score of mucosa was 22 out of 120 with a mean of 4.44 +/- 6.4.
- The maximum PDAI total score was 82 out of 263 with a mean of 10.82 +/- 13.16.
- PDAI score was zero in 19 patients who were in clinical remission.

- There were 16 patients (20.8%) who presented with only mucosal lesions.
- Forty two patients (54.5%) presented with both mucosal and cutaneous lesions.

Grading of PDAI score:

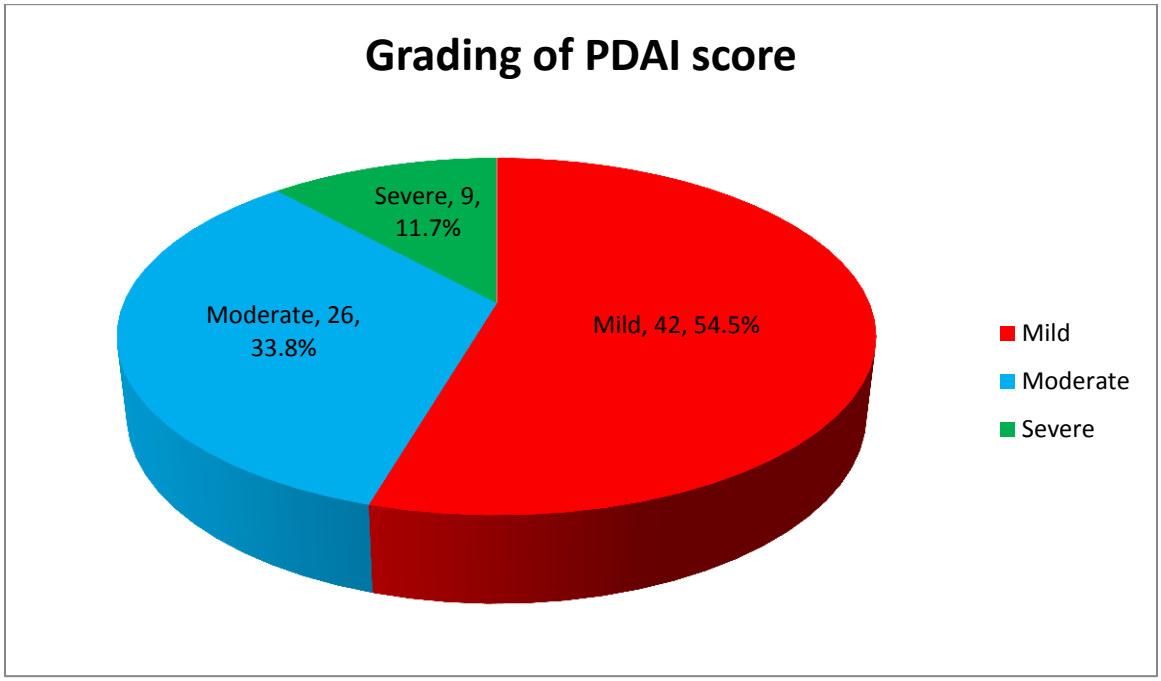


Figure 14 - The pie chart depicts the grading of disease according to PDAI score.

The chart (fig.14) shows that there were about 42 patients (54.5%) with mild disease, 26 patients (33.8%) with moderate disease and only 9 patients (11.7%) with severe disease

Tzanck smear:

Tzanck smear done from a vesicle or an erosion was positive for acantholytic cells in all the patients studied.

Direct immunofluroscence test:

Direct immunofluroscence test of the perilesional skin was done in all the patients and all of them showed IgG and C3 deposition in the intercellular region of lower epidermis with fish net appearance.

Treatment:

Majority of the study patients (72 patients), at presentation to our hospital were already on treatment. Among patients on treatment, 69 of them were on oral steroid therapy. Twenty patients were on steroid monotherapy. The range of daily dose of steroid varied from 10mg to 80mg.The mean daily dose of oral steroid was 12.7mg +/- 9.9.

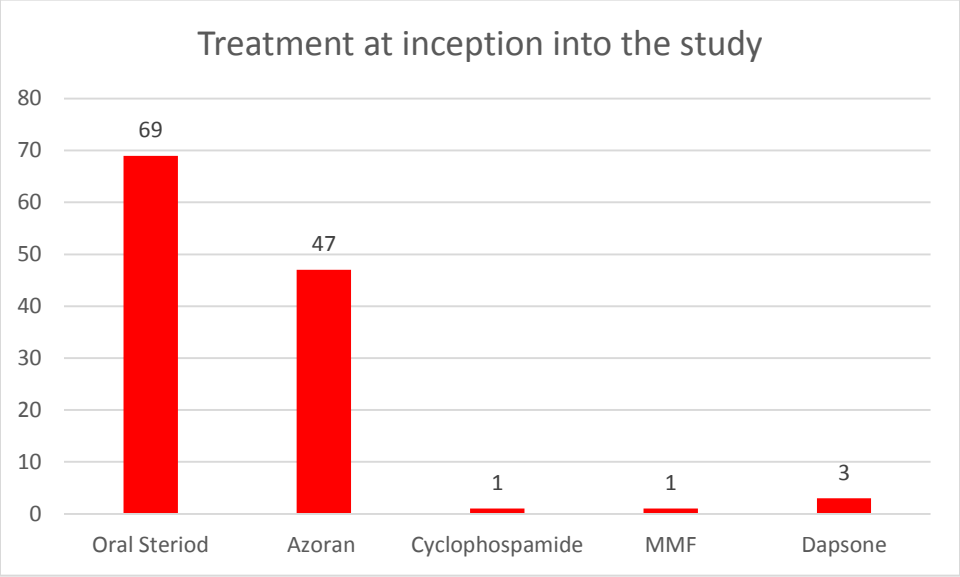


Figure 15 - Treatment at inception into the study

The chart (fig. 15) shows that majority of the patients (52 patients) were on other adjuvant drugs as well.

The adjuvant drugs used were

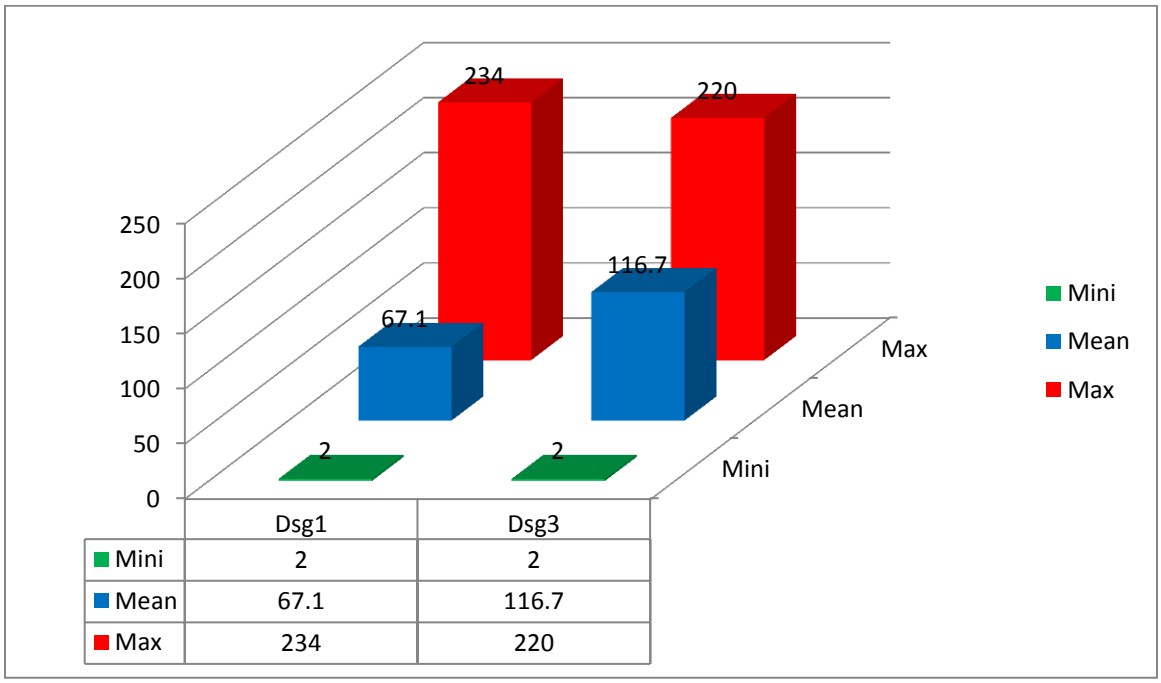
Azathioprine – 47/77 (61%), maximum dose – 150mg, mean dose – 45.11mg +/- 17.07mg.

Dapsone – 3/77 (4%), maximum and mean dose – 100mg

Mycophenolate mofetil – 1/77 (1%)

Cyclophosphamide – 1/77 (1%)

Range of desmoglein titres:



	Dsg1	Dsg3
Mini	2	2
Mean	67.1	116.7
Max	234	220

Figure 16 - Range of Dsg1 and Dsg3 titres.

Dsg1titres:- The graph (fig. 16) shows that the titres of desmoglein 1 ranged from minimum of 2 to maximum of 234 with a mean value of 67.1 +/- 70.8. The cut off value taken for the diagnosis of pemphigus vulgaris for both Dsg1 and Dsg3 titre was 20 u/ml. About 33 patients had low Dsg1 titres, among them 24 patients were in clinical remission and 8 of them had only mucosal lesions.

Dsg3 titres:- The graph (fig.15) shows that the titres of desmoglein 3 ranged from minimum of 2 to maximum of 220 with a mean value of 116.7 +/- 69.3. Desmoglein 3 titres of 12 patients (15%) were less (<20u/ml) than the cut off value. Of the patients with low Dsg3 values, 9 patients were in clinical remission and 3 patients had only cutaneous lesions.

Distribution of Dsg1 and Dsg3 titre in the study population:

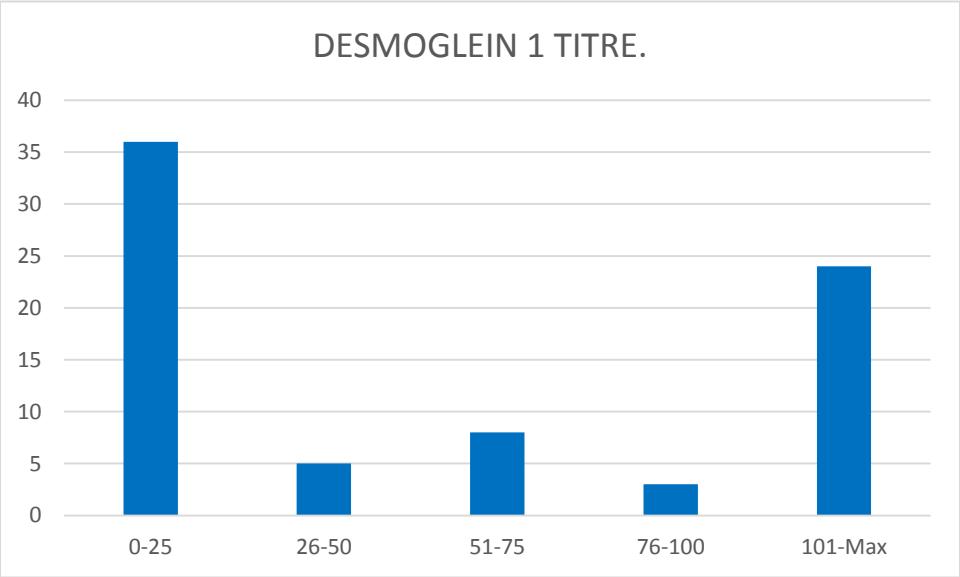


Figure 17 Distribution of Dsg1 in the study population.

The graph (fig.17) shows distribution of Dsg1 titres in the study population. Majority of them had low Dsg1 titres (<25u/ml) in the study population. It ranged from minimum of 0 to maximum of 234 u/ml.

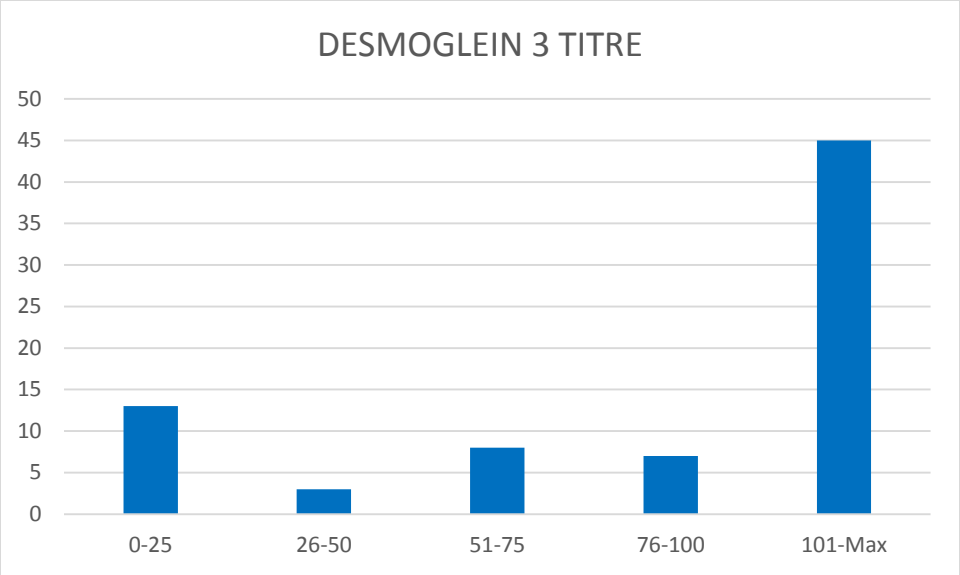


Figure 18 Distribution of Dsg3 in the study population.

The graph (fig.18) shows that most of the study population had high titres of Dsg3 (>100u/ml). There were 65 patients with positive Dsg3 titre, out of which 45 patients had Dsg3 value of more than 100 u/ml

Severity of the disease and the mean value of Dsg1 and Dsg3:

Grading of PDAI score	Mean value of Dsg1 antibody u/ml	Mean value of Dsg3 antibody u/ml+/-SD	Gender distribution Male : female
Mild	41.7 +/-60.5	91.9 +/-67.7	13:29
Moderate	97.4 +/-73.9	135.9 +/-63.9	10:16
Severe	109.18 +/-55.6	179.2 +/-22.4	1:8

Table 1 Shows the mean value of Dsg1 and Dsg3 antibody and its gender distribution in mild, moderate and severe disease.

As depicted in table 1 there is female preponderance in all the severity group of diseases. The mean value of Dsg1 and Dsg3 is increasing according the severity of disease.

Clinical subtypes of pemphigus vulgaris and its Dsg titres:

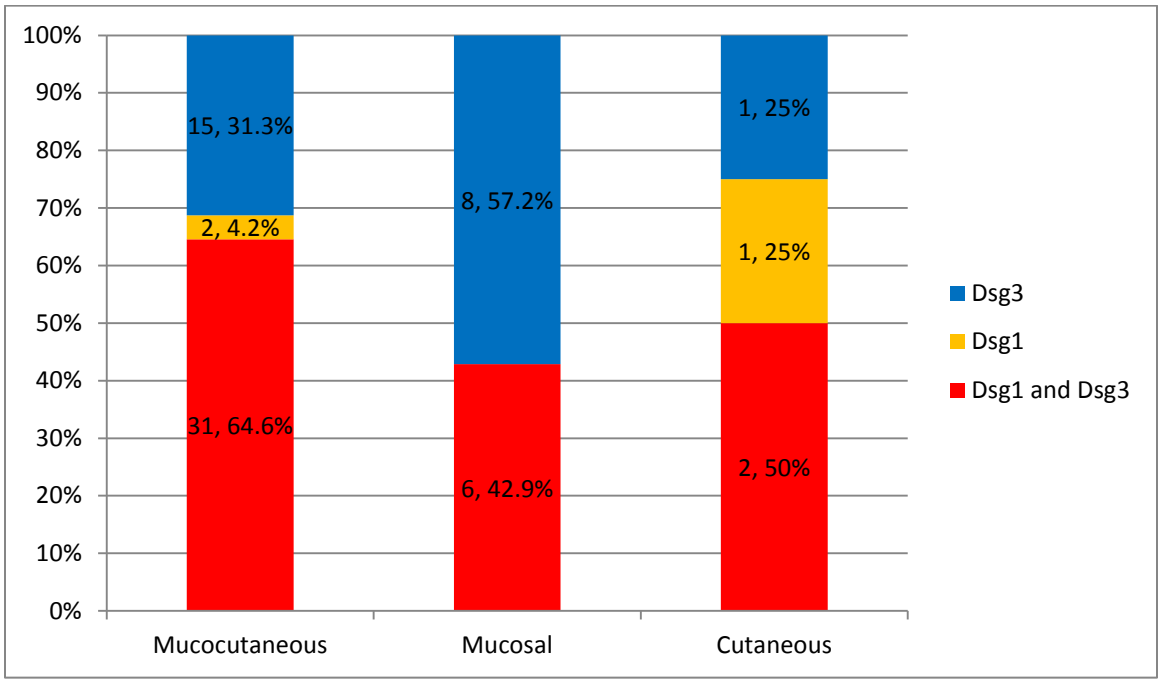


Figure 19 - Distribution of Dsg1 and Dsg3 titres in the clinical subtypes of pemphigus vulgaris.

Mucocutaneous subtype(n=56):

The bar chart (fig.19) shows that in mucocutaneous type of pemphigus vulgaris,

- 31patients (64.6%) had raised titres for both Dsg1 and Dsg3,
- 15 patients(31.3%) had elevated titres of only anti Dsg3 antibody,
- Isolated anti Dsg1antibody titre was raised in 2 patients (4.2%).

Mucosal subtype(n=15):

The bar chart (fig.19) shows that in mucosal subtype of pemphigus vulgaris,

- 8 patients (57.2%) showed raised titres of anti Dsg3 antibody alone,
- Both Dsg1 and Dsg3 antibody titre was raised in 6 patients(42.9%),
- None of the patients were found to have raised anti Dsg1 antibody titre.

Cutaneous subtype(n=6):

The bar chart (fig.19) shows that in cutaneous subtype of pemphigus vulgaris,

- 2 patients had raised titres of both anti Dsg1 and anti Dsg3 antibody titre,
- One patient each had isolated raised titres anti Dsg1 and anti Dsg3 antibody titre.

Mean value of anti Dsg3 and Dsg1 antibody titre in different subtypes of pemphigus is depicted in table.2and table.3

Subtype of pemphigus	Mean value anti Dsg3 antibody (u/ml) +/-SD	
Mucocutaneous	123.5 +/-67.9	p-value - 0.0224
Mucosal	127.0 +/-62.2	
Cutaneous	28.8 +/-37	

Table -2Shows the mean value of anti Dsg3 antibody among subtypes of pemphigus.

The table (Table 2) shows that the mean value of anti Dsg3 antibody titre was raised in mucocutaneous and mucosal type of pemphigus with a p-value of 0.0224 which is statistically significant.

Subtype of pemphigus	Mean value anti Dsg1 antibody (u/ml) +/-SD	p - value – 0.2075
Mucocutaneous	66.28 +/-68.4	
Mucosal	54.03 +/-78.1	
Cutaneous	106.6 +/-71	

Table -3 Shows the mean value of anti Dsg1 antibody among subtypes of pemphigus.

The table (Table 3) shows that the mean value of anti Dsg1 antibody titre is raised in cutaneous type of pemphigus when compared with mucocutaneous and mucosal subtypes. But this did not reach the level of statistical significance (p-value=0.20).

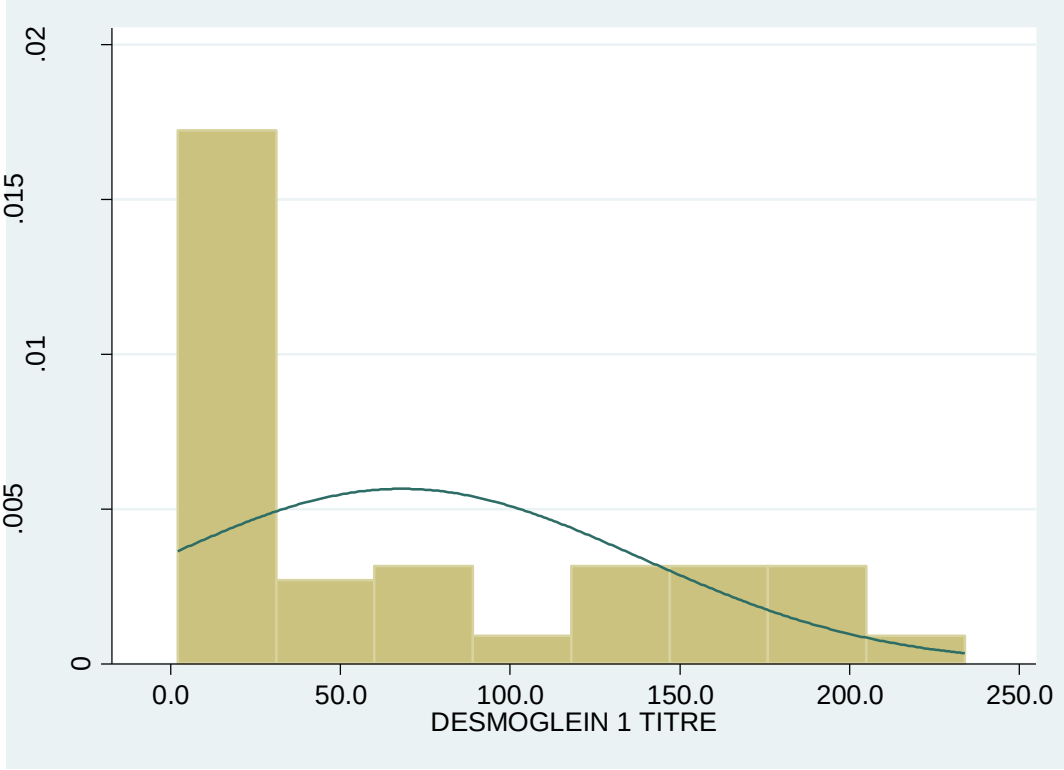


Figure 20 Histogram depicting distribution of Dsg1 in the study population.

The histogram (fig:20) does not follow a normal curve. It denotes that there is predominance of low titre of Dsg1 in the study population

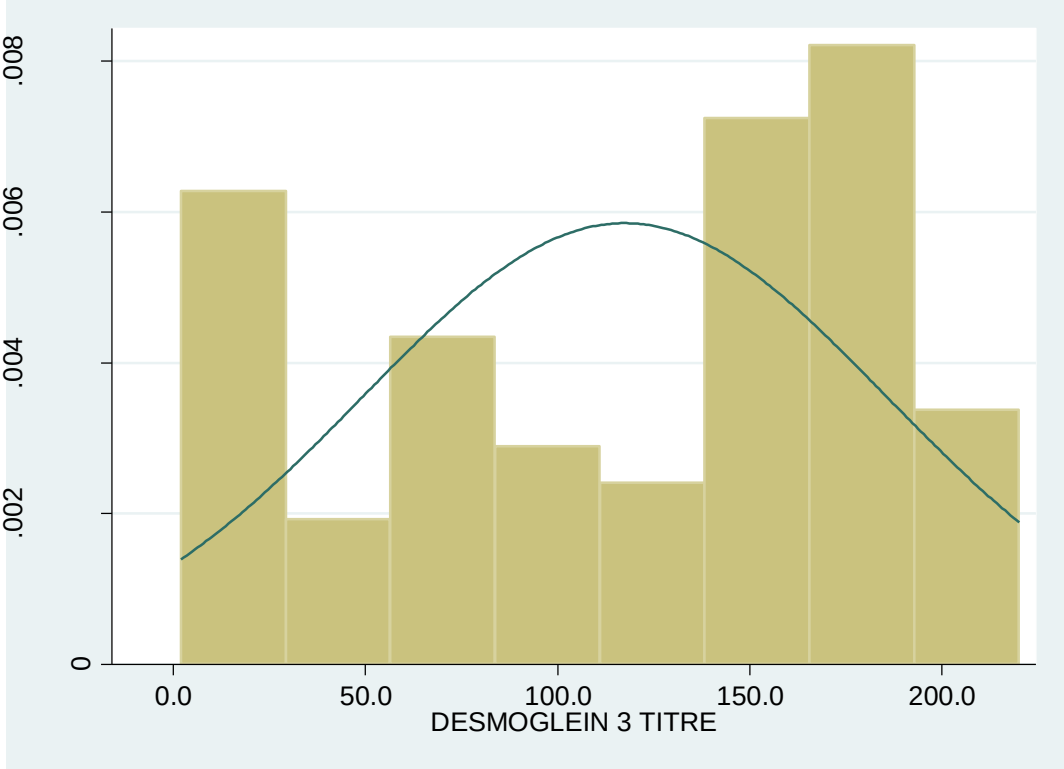


Figure 21 Histogram depicting distribution of Dsg3 titre in the study population

The histogram (fig. 21) does not follow a normal curve. It denotes that there is predominanace of high titre of Dsg3 antibody in the study population.

Correlation PDAI score with anti Dsg1 titres in the study population:

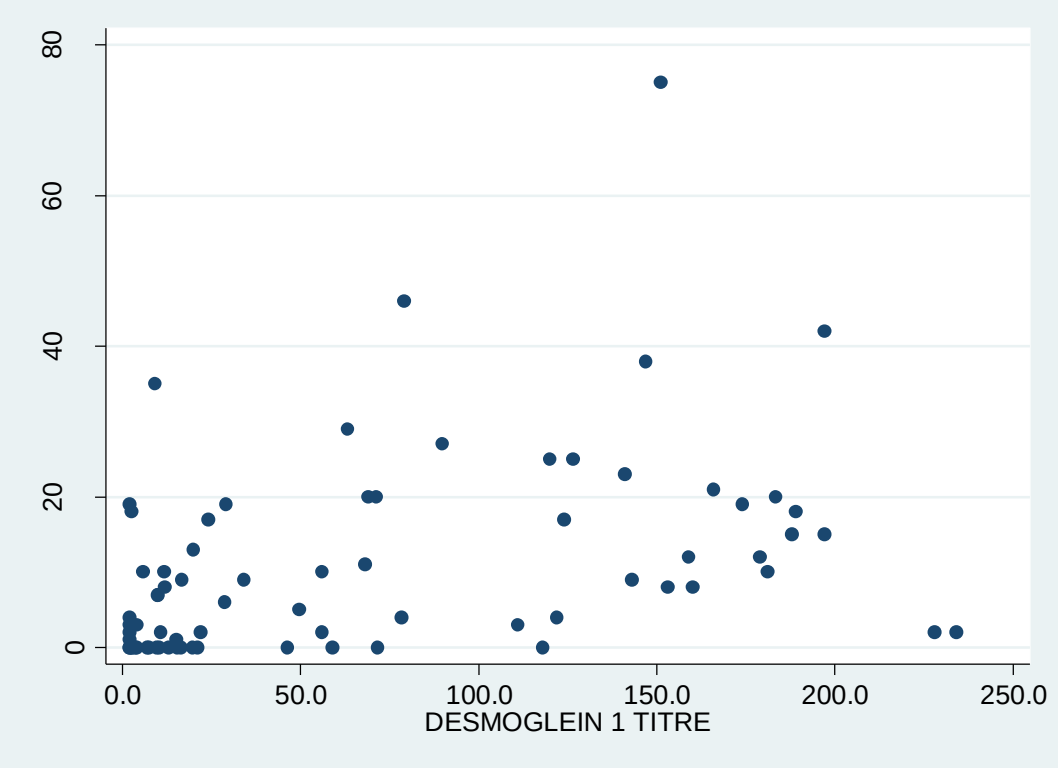


Figure 22 Scatter plot compares total PDAI score with anti Dsg1 titre.

Table 4 Spearman's correlation of coefficient of anti Dsg1titre with PDAI score.

		PDAI
Dsg1	Correlation coefficient	0.4
	p-value	<0.01

The scatter plot (fig.22) and table 4 compares PDAI score with anti Dsg1 antibody titre and shows a correlation co-efficient of 0.4 and p-value of <o.o1 which is statistically significant. There is a rising trend in the scatter plot as the PDAI score and Dsg1 titre increases.

Correlation PDAI score with anti Dsg3 titres in the study population:

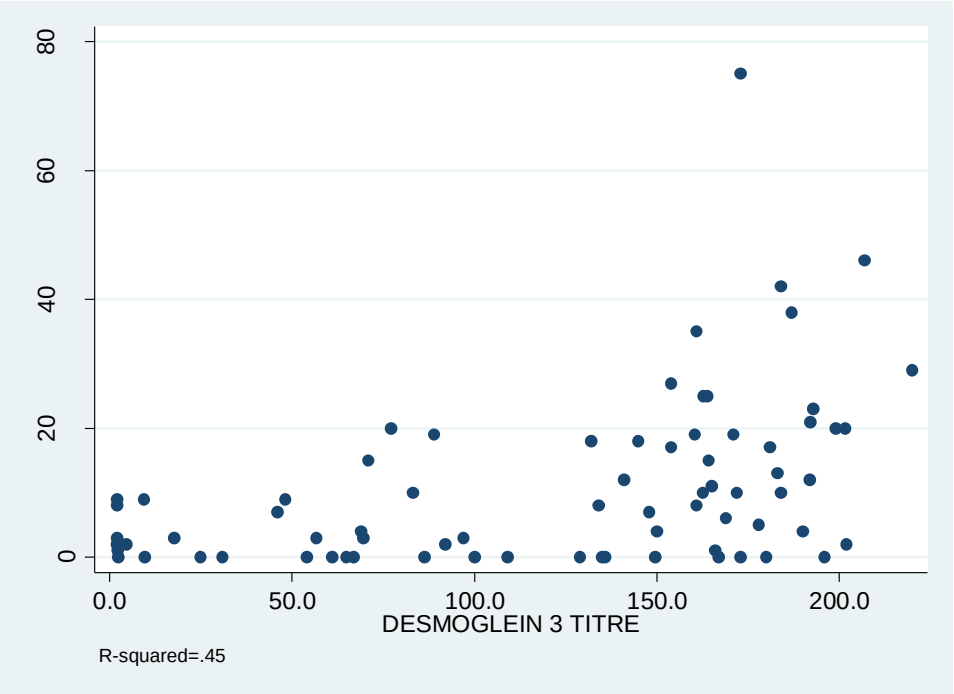


Figure 23 Scatter plot compares PDAI score with Dsg3 titres in the study population.

		PDAI
Dsg3	Correlation coefficient	0.43
	p-value	<0.01

Table 5 Spearman's correlation coefficient of anti Dsg3 titre with PDAI score.

The scatter plot (fig.23) and table 5 shows the comparison of anti Dsg3 antibody titre with PDAI total score and has a correlation coefficient of 0.43 and a p-value of <0.01 which is statistically significant. The scatter plot shows a rising trend as the titre of Dsg3 and PDAI score increases.

Acetylcholine receptor antibody titre:

There were a total of 17 patients (22%) with raised AChRAb titre. The prevalence of AChRAb in our study population was 22%.

Characteristics of AChRAb positive patients:

S.No.	Age in years	Sex	Duration of illness in months	Subtype	PDAI total score	On treatment at presentation	Dsg1 and Dsg3
1	31	F	12months	mucocutaneous	10	yes	↑Dsg3
2	79	M	1month	mucocutaneous	15	yes	↑Dsg1 and Dsg3
3	40	F	18 months	mucocutaneous	38	yes	↑Dsg1 and Dsg3
4	45	F	96 months	Cutaneous	8	yes	↑Dsg1
5	49	F	96 months	mucocutaneous	11	yes	↑Dsg1 and Dsg3
6	56	F	1 month	mucocutaneous	46	no	↑Dsg1 and Dsg3
7	43	M	36 months	mucosal	13	yes	↑Dsg1 and Dsg3
8	51	F	60 months	mucocutaneous	4	yes	↑Dsg3
9	47	M	1month	mucocutaneous	17	yes	↑Dsg1 and Dsg3
10	64	M	12 months	mucocutaneous	3	yes	↑Dsg3
11	23	F	36 months	mucocutaneous	0	yes	↑Dsg1 and Dsg3
12	34	M	96months	cutaneous	9	yes	Negative
13	70	F	60 months	mucosal	0	yes	↑Dsg3
14	56	F	24 months	mucocutaneous	0	yes	↑Dsg3
15	54	M	72months	mucocutaneous	1	yes	↑Dsg3
16	46	F	36 months	mucocutaneous	0	yes	↑Dsg3
17	31	M	4months	mucocutaneous	21	yes	↑Dsg1

Table 6 Shows the characteristics features of AChRAb positive patients.

The table 6 shows the characteristic features of AChRAb positive patients. There were 13 patients of age >40yrs. The duration of illness ranged from minimum of 1 month to maximum of 96 months. All the patients were on treatment at the time of presentation except one patient. All the AChRAb positive patients had raised titres of either Dsg1 or Dsg3 except one.

Gender distribution among positive patients:

Total number of patients	17
Male	7
Female	10

Table. 7 Gender distribution in AChRAb titre positive patients.

The table (table.7) shows the gender distribution among AChRAb titre positive patients with female preponderance.

Range of acetylcholine receptor antibody titre in the positive patients:

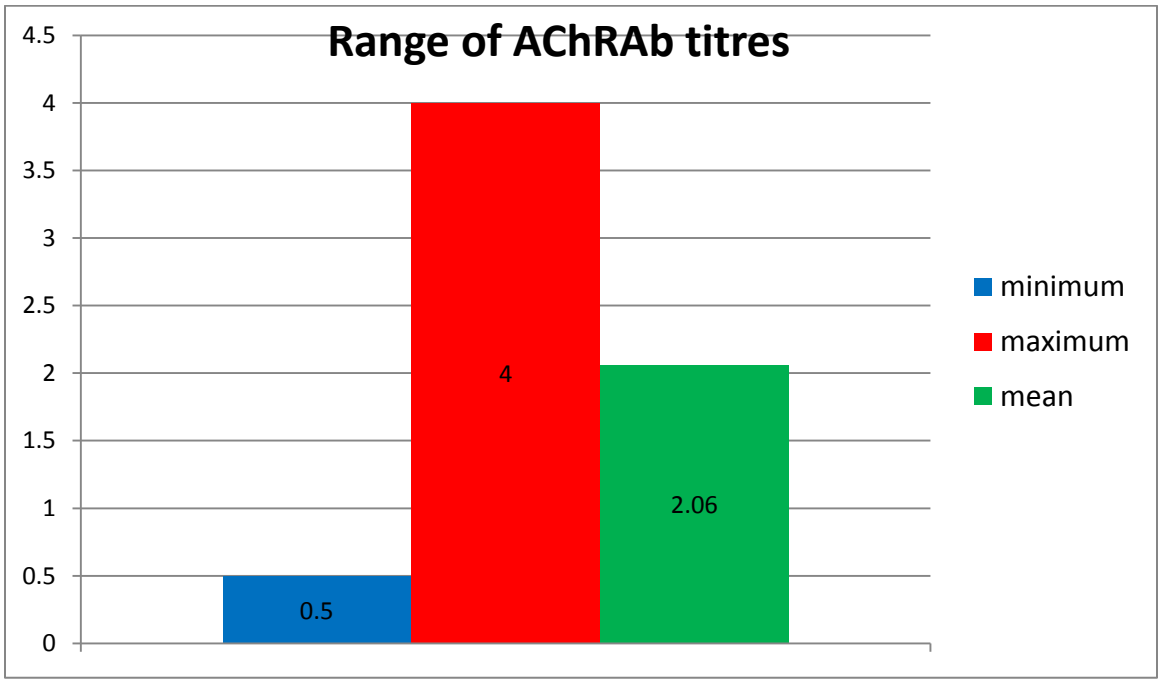


Figure 24- Range of AChRAb titre in the positive patients.

The graph (fig.24) depicts the range of AChRAb titres in the positive patients. The range varied from a minimum of 0.5nmol/l to a maximum of 4.0nmol/l. The minimum cut off value for acetylcholine receptor antibody titre was taken as ≥0.45nmol/l The mean value of AChRAb titre is 2.06nmol/l +/- 1.1nmol/l.

Among AChRAb titre positive patients, 13 patients were of mucocutaneous type and 2 each of mucosal and cutaneous type.

Subtypes in AChRAb positive patients:

Subtype of pemphigus	Mean (nmoles/l)+/-SD
Mucocutaneous – 13/17	2.2 +/-1.1
Mucosal – 2/17	2.6 +/-0.7
Cutaneous – 2/17	0.8 +/-0.4

Table.8 Mean value of AChRAb titre in subtypes of pemphigus vulgaris patients.

The table.8 depicts the mean value of AChRAb titre among positive patients.

Grading of disease severity and corresponding mean value:

Severity of disease	Mean (nmoles)/l) +/-SD
Mild – 8/17	2.4+/-1.0
Moderate – 7/17	1.9+/-1.2
Severe – 2/17	1.1+/-0

Table.9 Mean value of AChRAb titre and disease severity.

The table.9 portraits the mean value of AChRAb titre and disease severity. Among AChRAb titre positive patients, 8 patients had mild disease, 7 patients had moderate disease and 2 patients had severe disease.

AChRAb positive patients and their desmoglein titres:

Among AChRAb positive patients, there were 7/17 patients (10.4%) with elevated titres of Dsg3 antibody and 8/17 patients with elevated titres with both Dsg1 and Dsg3 antibody titre. Isolated raised Dsg1 titre was seen only in one patient with AChRAb positivity.

		PDAI	Dsg3	Dsg1
AChRAb	Correlation co-efficient	0.03	0.025	0.09
	p-value	0.76	0.52	0.7

Table.10 Spearman's correlation coefficient of AChRAb titre with PDAI score, anti Dsg1 and anti Dsg3 titre

The table.10 depicts the correlation of AChRAb titre with PDAI total score, anti Dsg1 antibody titre and anti Dsg3 antibody titre. This was not statistically significant.

CLINICAL PHOTOGRAPHS OF PEMPHIGUS VULGARIS PATIENTS

Figure. 25 **C**rusted erosions over chest, neck and abdomen, (PDAI - 42).

Figure. 26 and 27 Crusted erosions over chest, abdomen, back and upper extremity,

(PDAI - 35).

Figure. 28 Erosion with re-epithelialisation and acanthoma,(PDAI - 30).

Figure. 29 Erosion over periocular, perioral, labial and palatal mucosa.

Figure. 30 Crusted erosion over scalp.

Figure. 31 Erosion over buccal mucosa.

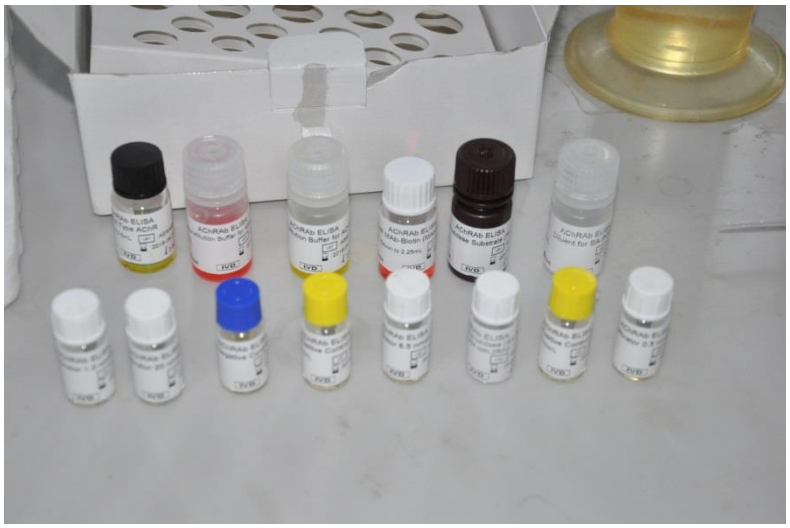


Figure. 32 Reagents used in AChRAb ELISA kit

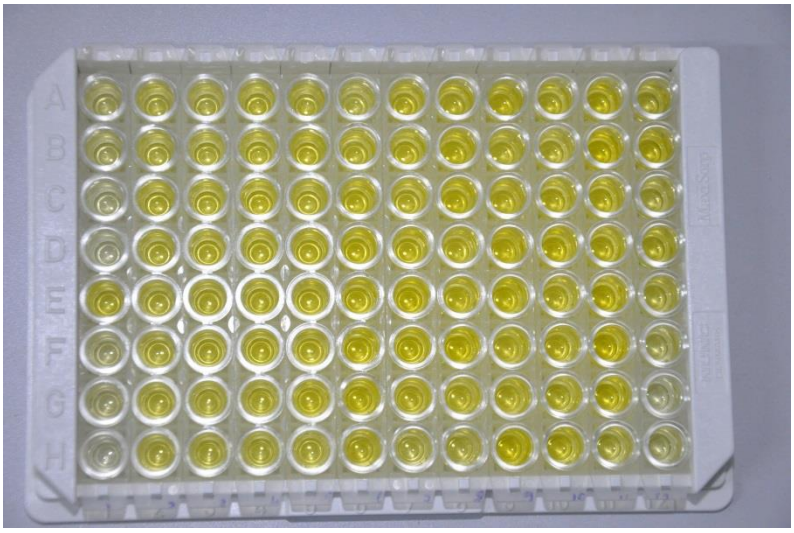


Figure. 33 AChRAb ELISA plate with samples.

DISCUSSION

Pemphigus vulgaris is a chronic autoimmune intraepidermal bullous disorder. The incidence rate of pemphigus vulgaris among dermatology outpatient attendees in India is 0.09% - 1.8%(3)(18). Clinically pemphigus is characterised by flaccid blisters and erosions of skin and mucosa. Autoantibodies directed against desmoglein adhesion molecules, Dsg1 and Dsg3 leads to detachment of keratinocytes in the epidermis resulting in the process of acantholysis. Stanley et al. reports that these anti desmoglein antibodies are sufficient for the formation of blisters in pemphigus patients(12). Mahoney et al. and Udey et al. also states that both Dsg1 and Dsg3 antibodies are essential for the process of acantholysis in pemphigus(12). Kumar et al. estimated Dsg1 and Dsg3 antibodies titres by ELISA method and correlated it with disease severity(9). He observed high titres of Dsg3 in patients with severe oral mucosal involvement and high titres of Dsg1 in case of cutaneous involvement regardless of type of pemphigus(57). But majority of patients in remission also had high titres of Dsg3 antibody. This points out that the estimation of titre of pathogenic IgG subclass is more valuable in assessing the disease severity instead of whole of IgG titre. He also observed low titres of Dsg1 and Dsg3 in clinically active pemphigus(17). This was explained due to the presence of pathogenic antibodies against non desmoglein antigens or against the intracellular domain of Dsg1/Dsg3 which is undetectable by ELISA kit. Grando et al. recently listed a group of autoantigens which includes adhesion molecules (Dsg1, Dsg2, Dsg3), plakoglobulins, desmocollins, BP180 and receptor molecules like alpha9 AChR, alpha3 AChR, pemphaxin and other annexins, FcεRIα to be involved in the pathogenesis of

pemphigus(45). In addition Nguyen et al. concluded that antibodies against non desmoglein antigens on the surface of keratinocytes can induce pemphigus vulgaris like lesions(12). These non desmoglein antibodies were identified as acetylcholine receptor antibodies involved in regulation of keratinocytes. Identification of these antigens led to the search of novel therapies like cholinomimetics to ameliorate the side effects of corticosteroid. Grando and Dahl observed antiacantholytic effect of cholinomimetics on keratinocytes in vitro(12)(65).There is only a single published study on correlation of acetylcholine receptor antibodies with desmoglein titres and pemphigus disease severity(66). There are no published data on acetylcholine receptor antibody in pemphigus from India.

Hence, we studied the correlation of disease severity with titres of acetylcholine receptor antibodies and desmoglein titres in pemphigus patients attending our hospital. Pemphigus disease area index clinical score was used to quantify the disease severity. The diagnosis of pemphigus was made based on clinical features, classical histopathological finding of suprabasal bulla and direct immunofluroscence of the perilesional skin showing IgG and C3 deposition in the intercellular region of epidermis having a fishnet pattern. Seventy seven pemphigus patients who attended the dermatology department with either active disease or in remission during the study period, were included in the study.

Demographic profile:

Age of the patient at presentation:

Majority of the patients (70%) were above 40 yrs which is similar to that of other studies(67)(68). However, the data from India showed a different trend. Mascarenhas et al. and Singh et al. observed that more than 50% of pemphigus patients were seen below the age of 40yrs(17)(18).

The mean age of onset of the disease in our patients studied was 46yrs. Similar observation was seen in other studies, where the mean age of presentation of pemphigus vulgaris was above 40 yrs.(69–71) The youngest patient in our study was 22yrs old and the oldest was 79yrs old.

Gender distribution:

The male:female ratio in our study was 1:2.2 with female preponderance. Similarly female preponderance was observed in other studies too(69–72). However, some studies showed a male preponderance(3)(18)(73).

Duration of disease:

The average duration of disease ranged from minimum of 2 months to 18 yrs.

Site of lesion:

The commonest site of onset of cutaneous involvement was the chest and buccal mucosa was the commonest mucosal site of onset in our patients.

In the oral cavity, majority of patients (36 patients) had buccal mucosal involvement followed by tongue, palate, labial and gingiva. Oral lesions are most common at the site of frictional trauma like buccal mucosa followed by tongue, palate and labial mucosa(71). Other mucosa involved were nasal and genital mucosa in 3 of our patients.

Clinical subtype of pemphigus:

The commonest subtype of pemphigus vulgaris seen in our study population was mucocutaneous type (73%). This was followed by mucosal (15%) and cutaneous (5%). This is in concordance with other studies, in which they observed more than 50% of patients with mucocutaneous type of pemphigus vulgaris(1)(74)(75). Kavusi et al. in his retrospective study has found that mucocutaneous subtype of pemphigus patients has a lower rate of remission and frequent rate of relapses(76).

Pemphigus disease area index:

Pemphigus disease area index is a clinical scoring system which measures the extent of cutaneous and mucosal lesions. Pemphigus disease area index is a validated scoring system with reasonable convergent validity. It is easy, quick and reliable method and correlates better and is more reproducible(58). It has high intra-rater reliability than ABSIS clinical scoring. The minimum score of PDAI was zero for all the sites. Maximum score for skin in our patient was 50 out of 132 with a mean of 5.3 +/- 8.6. Similarly maximum score of mucosa was 22 out of 120 with a mean of 4.44 +/- 1.64. The maximum score of scalp was 10 out of 11 with a mean of 1.08 +/- 2.08. Finally maximum PDAI total score was 82 out of 263 with a mean of 10.82 +/- 13.16.

Shimizu et al. in his study in pemphigus patients compared PDAI with JPDSS(Japanese pemphigus disease severity score) and physician's subjective impression and proposed grading criteria for PDAI. After studying 37 pemphigus patients and analyzing 110 assessments he graded PDAI as mild (0-8), moderate (9-24) and severe (≥25).He concludes that PDAI is the excellent scoring system to evaluate pemphigus disease severity(77).

In our study, mild disease was seen in 42 patients, moderate disease in 26 patients and severe disease in 9 patients. Majority of our patient (55%) had mild disease.

Treatment:

At the time of initial presentation 93% of our patients were already on treatment, of which 90% patients were on combination of oral steroid and adjuvant therapy and about 20 patients were on steroid monotherapy. Steroid was used as first line of treatment for all the patients. The advent of steroid has reduced mortality from 90% to 10% in pemphigus patients(78). Azoran which is the main steroid sparing agent used in pemphigus was used for 60% of our patients. Daniel and Murrell in a randomized controlled study on 58 patients compared the efficacy between a combination of steroid with azathioprine and steroid with placebo. At the end of 1 yr of treatment there was no statistically significant difference between the two groups. But azathioprine showed significant steroid sparing effect. In another RCT, he compared the efficacy of azathioprine with mycophenolate mofetil and cyclophosphamide and found similar efficacy in all the three(78). However the first line adjuvant drug considered are

azathioprine and mycophenolate mofetil(78). Three of our patients were also on dapsone and one each on mycophenolate mofetil and cyclophosphamide. In a four arm study, Chams-Davatchi et al. compared the efficacy of steroid monotherapy, steroid with azathioprine, steroid with mycophenolate mofetil and steroid with intravenous cyclophosphamide and concluded that the efficacy of steroid is increased when given along with an adjuvant. He considered azathioprine as the first line adjuvant followed by cyclophosphamide(79). Anti CD 20 human monoclonal antibody, rituximab seems to be a promising drug for treatment resistant pemphigus. Schmidt et al in his study on 26 treatment resistant patients observed clinical improvement in all except one when treated with rituximab(62).

Tzanck smear test:

Tzanck test was positive in all patients. It is considered as a useful bedside test in the diagnosis of pemphigus vulgaris(52)(54). It is especially useful as a bedside test to rule out clinical presentations mimicking pemphigus like bullous pemphigoid and bullous SLE.

Direct immunofluroscence (DIF):

Direct immunofluroscence test done in all the patients was positive for IgG and C3 in the intercellular region having a fish net pattern in the lower epidermis. It is a reliable diagnostic test and shows positivity in early course of the disease of pemphigus patients(80). Sethi et al. in his prospective study of 20 pemphigus patients on DIF concludes that positive DIF is an indicator of imminent relapse(81). In a study of 57

patients of pemphigus vulgaris in clinical remission, Balighi et al. concludes that pemphigus vulgaris patients in 6-12months of clinical remission with negative DIF is a useful indicator of immunological remission(82).

Distribution of desmoglein titres in the subtypes of pemphigus:

Mucocutaneous subtype (n=55): In mucocutaneous subtype of pemphigus majority of patients (64.6%) had raised titres of both anti Dsg1 and anti Dsg3 antibody titre. Isolated anti Dsg1 antibody elevation was found in 4.2% of patients and isolated anti Dsg3 antibody elevation was found in 31.3% of patients.

Mucosal subtype (n=15): In mucosal type of pemphigus, 57.2% had isolated anti Dsg3 antibody titre elevation and 42.9% had raised titres of both anti desmoglein 1 and anti desmoglein 3 antibody and none were found to have raised titres of anti Dsg1 antibody titre.

Cutaneous type (n= 6): In cutaneous type, both anti Dsg1 and anti Dsg3 antibody value were raised in 2 patients. Isolated elevation of anti Dsg1 and anti Dsg3 titre were seen in one patient each.

In our study, the clinical subtype of pemphigus correlated well with Dsg antibody profile except in few cases. Both Dsg 1 and Dsg 3 were found to be elevated in majority of patients with mucocutaneous type and only anti Dsg3 antibody titre was raised in majority of patients with mucosal lesion. Similarly Daneshpazhooh et al. and Amagai et al. in their study observed that clinical subtype of pemphigus correlated with Dsg antibody profile (Mucosal type - only Dsg3 antibody was positive, Mucocutaneous type -

both Dsg1 and Dsg3 antibody was positive)(10)(83). However, there are few reported cases with discrepancies in the desmoglein antibody profile and clinical phenotype observed(10). This was thought to be due to either genetic variations in the patients or presence of minor antigens involved in the pathogenesis of pemphigus patients(10).

Mean value of anti Dsg1 and Dsg3 antibody among subtypes of pemphigus:

The mean value of anti Dsg3 in our study patient were higher in mucosal and mucocutaneous subtype which was statistically significant (p-value=0.02). Though the mean value of Dsg1 antibody titre was raised in cutaneous subtype, this was not statistically significant (p-value=0.2). Daneshpazhooh et al. in his study of 73 pemphigus patients, observed that the mean index value of Dsg1 antibody was found to be higher in mucocutaneous and cutaneous subtype than mucosal type. He also observed that the mean index value of Dsg3 antibody titre was lower in cutaneous and mucosal subtype than mucocutaneous type(10).

Range of desmoglein titres:

Dsg1 titre: The desmoglein1 titres ranged from minimum of 2 to maximum of 220u/ml with a mean of 117.43+/-70.45. There is predominance of low titres of Dsg1(<25u/ml) in the study population. In about 33 patients Dsg1 titres (43%) were less than the cut off value and among them 24 patients were in clinical remission and 8 of them had mucosal lesions only.

Dsg3 titre: The range of desmoglein3 titres varied from minimum of 2 to maximum of 234 with a mean of 67.99 +/- 68.19. Majority of our study population (83%) had high titres(>100u/ml) of Dsg3. In about 12 patients (15%), Dsg3 titres were less than the cut off value. Out of the 12 patients with low Dsg3 titres, 9 patients were in clinical remission and 3 patients had only cutaneous lesions.

There were about 39 patients (50%) with raised titres of both Dsg1 and Dsg3. About 24 patients (31%) had elevated titres of anti Dsg3 antibody only. There were about 3 patients (4%) with raised titres of Dsg1titre only.

The above data clinically correlated well with our clinical phenotypic profile of our patients. Majority of them have raised titres of both Dsg1 and Dsg3 which corresponds to mucocutaneous type of pemphigus and it was the most predominant type seen in our study. This is in contradiction to the study of 26 pemphigus patients by Sharma et al. where he found that Dsg1 and Dsg3 antibody titre did not differentiate between different clinical subtypes of pemphigus(57).

Desmoglein titres and PDAI:

Amagai et al. studied the practical use of Dsg1 and Dsg3 ELISA assay in a large number of serum samples. He observed Dsg3 ELISA titre was positive in 79/80 patients of pemphigus vulgaris and 48/49 patients of pemphigus foliaceous were positive for Dsg1 titre. He also found that Dsg titre fluctuated with disease activity in 3 of its samples. He thus concluded that Dsg1 and Dsg3 ELISA titre is a sensitive and specific test for the

diagnosis of pemphigus and can be used as a tool to monitor disease activity(84)(85). Similar observation were noted in an Iranian and an Indian study(10) (86).

Abasq et al. in a retrospective study of 27 patients observed that the value of anti Dsg1 antibody correlated well with disease course. However there was no correlation with anti Dsg3 antibody value(87).

On the contrary, Bellon et al. in his study of 32 patients, evaluated anti desmoglein antibody titre by ELISA and followed up pemphigus patients and compared with DIF and indirect immunofluroscence in patients with complete remission and observed that Dsg ELISA is not useful for immunological monitoring of pemphigus patients(88).

In our study both Dsg1 and Dsg3 titre correlated well with disease activity, measured by PDAI clinical scoring and this was found to be statistically significant (p-<0.01). Hence desmoglein titres can be used as a valuable tool to monitor disease activity in pemphigus patients. More follow up studies comparing desmoglein levels and disease severity needs to be done to reach a definite conclusion.

Estimation of acetylcholine receptor antibody titre:

There were 17 patients (22%) who were found to be positive for acetylcholine receptor antibody titre. The calculated mean value of AChRAb is 2.06+/-1.1. The range of AChRAb titres among the positive patients varied from minimum of 0.5 to maximum of 4.0nmol/l. Among 17 patients, there were 10 females and 7 males.

Correlation of AChRAb with PDAI grading:

Among 17 patients with positive AChRAb, 8 patients had mild disease, 7 patients had moderate disease and 2 patients had severe disease. Among AChRAb positive patients, the PDAI total score ranged from minimum of 0 to maximum of 46.

Correlation of AChRAb with desmoglein titres:

Among the AChRAb positive group, there were 7 patients (10.4%) with raised Dsg3 antibody titre and another 8 patients (10.4%) with raised titres of both Dsg1 and Dsg3 antibody. One patient (1%) had isolated Dsg1 elevation. Dsg1 and Dsg3 antibody titre was negative in one patient.

Sanchez et al. in his study of 31 recently diagnosed pemphigus patients , observed that there is mild raise in acetylcholine receptor antibody titre M3 in all the patients and correlated well with disease severity measured by BSA and anti Dsg3 titre at the initial presentation and follow up. Baseline AChRAb titre was 1.5nmol/l and decreased to 0.5nmol/l during the follow up. Finally he concluded that acetylcholine receptor antibody M3 titre and anti desmoglein3 antibody titre can be used for disease monitoring at the initial and follow up time(11).

Acetylcholine antibodies were positive only in 22% of our patients. This low positivity could be attributed to the fact that most of our patients were on treatment and were in clinical remission. The presence of AchRab in 22% of our patients, may be a pointer that this antibody along with anti desmoglein antibodies would have played a role in the pathogenisis of pemphigus.

Grando and Dahl in their study demonstrated that muscarinic agonists like carbechol reverses acantholysis induced by pemphigus antibody in epidermal keratinocyte(65). Fania et al. in his study observed that acetylcholine receptor antibody is involved in the early stage of acantholysis and hence concludes that cholinergic agonist has a role to play in the treatment of pemphigus(49). Vu et al. in his study proved the pathogenicity of cholinergic receptor antibodies resulting in acantholysis in pemphigus patients. He injected pemphigus vulgaris IgG antibodies into genetically engineered Dsg3 null neonatal mice and these antibodies did not cross react with Dsg1. He noticed extensive skin blistering in the neonatal mice. He also used cholinergic receptors derived from human keratinocyte cell membrane as an antigen in radioimmunoprecipitation assay and observed precipitation of cholinergic receptors by the pemphigus autoantibodies thus proving its pathogenicity in pemphigus(42). Grando in his study concludes that there are two types of cholinergic receptors a) alpha9 acetylcholine receptor and b) pemphaxin involved in the pathogenesis of pemphigus and thus paving a way for the introduction of nonhormonal treatment for pemphigus. Grando postulates that antibodies in pemphigus patients are developed against different types of receptors in different patients(12). All these studies claims the role of AChRAb in the pathogenesis of pemphigus.

In comparison to study done by Sanchez et al. mentioned above the maximum value of AChRAb titre in our study is comparatively high. But in our study there was no statistical significant correlation of AChRAb titre with PDAI, Dsg1 and Dsg3.

The explanation to the poor statistical correlation of AChRAb with PDAI, Dsg1 and Dsg3 antibody titre may be attributed to the fact that majority of the patients had mild disease and were in remission at the time of inclusion into the study. Detection of newer cholinergic receptors like α9 acetylcholine receptor and pemphaxin involved in the pathogenesis paves the way for further study on the newer receptors mentioned above.

However further follow up of the patient to monitor AChRAb titre is essential for definite conclusion.

CONCLUSIONS

We observed the following conclusions in our hospital based pilot study on corelation of disease activity with AChRAb and Dsg1 and Dsg3 titre in pemphigus vulgaris.

1) The mean age of our patients was 46 yrs+/-13yrs
2) There was female preponderance in all the age groups. The male : female ratio was 1:2.2.
3) Patient was graded as mild, moderate and severe disease according to PDAI score and majority of them 42patients (55%) found to have mild disease.
4) Majority of patients (n=56, 75%) had mucocutaneous subtype of pemphigus vulgaris
5) Majority of patients (n=31, 64.6%) with mucocutaneous subtype had raised titres of both Dsg1 and Dsg3.
6) In mucosal subtype of pemphigus isolated elevation of Dsg3 titre was seen in 8 patients (57.2%)
7) Anti desmoglein1 antibody elevation was not seen in any patient with mucosal pemphigus.
8) The mean value of Dsg3 antibody was higher in mucosal and mucocutaneous subtype than cutaneous type. This was statistically significant (p-value-0.02).
9) The mean value of Dsg1 antibody titre in different subtype of pemphigus showed no statistical significance (p-value=0.2)

10) There was significant statistical correlation of PDAI score with anti Dsg1 and Dsg3 antibody titre.(p-value - <0.01)
11) The prevalence of acetylcholine receptor antibody in our study population was 22%.
12) The mean value of AChRAb titre in all the patients was 2.06+/-1.1.
13) There was no significant statistical correlation of AChRAb titre with disease severity measured by PDAI (p-value =0.76).
14) There was no significant statistical correlation of AChRAb titre with Dsg3 and Dsg1 antibody titre (p-value –0.52 and p-value=0.7 respectively).

Limitations of the study

1) Majority of the patient had mild disease and was in clinical remissions, hence the AChRAb value may not be representative.
2) Majority of the patients were on maintenance phase of treatment.
3) Antibodies against M3 acetylcholine receptor alone was evaluated, the recently detected cholinergic receptors like α9 acetylcholine receptor and pemphaxin was not evaluated.
4) There was no control group.

Recommendations

1) AChRAb titre estimation in the treatment naïve pemphigus patients and repeat estimation of the same after initiation of treatment and treatment induced remission.
2) AChRAb titre estimation in patients with active disease.
3) Cholinergic receptors like novel α9 acetylcholine receptor and pemphaxin are involved in the pathogenesis of pemphigus vulgaris. Hence estimation of AChRAb against these receptors are recommended for the future studies.
4) Control groups needs to be included in the study to look for the prevalence of these antibodies in general population.

SUMMARY

Background:

Pemphigus vulgaris is a chronic autoimmune bullous disorder with remission and exacerbation. It is characterized histopathologically by intraepidermal cleft due to antibodies against keratinocyte adhesion molecules in the epidermis called desmosomes. Desmoglein1 and desmoglein3 antibodies were involved in the pathogenesis of pemphigus. But Nguyen et al. concluded in his study that nondesmoglein antigens like cholinergic receptors on the surface of keratinocytes are involved in the pathogenesis. He also reported that 85% of pemphigus patients have antibodies against cholinergic receptors. This led to the novel search of nonhormonal drugs for the treatment of pemphigus to decrease the side effects of steroids. Desmoglein antibody titre is routinely estimated to diagnose and to monitor the disease severity of pemphigus patients. There are occasional cases in which discrepancy of desmoglein antibody titre and disease severity was noted.

Objective:

1) To estimate the prevalence of acetylcholine receptor antibodies in patients with pemphigus.
2) To correlate the serum anti acetylcholine receptor antibody titres with clinical disease activity as measured by the pemphigus disease area index (PDAI) in patients with pemphigus.
3) To compare the values of the serum anti acetylcholine receptor antibody titres with anti desmoglein antibody titres in patients with pemphigus.

Methodology:

We included 77 pemphigus patients in our study between a period of October 2014 to August 2015. At the time of initial presentation disease severity was calculated by PDAI scoring system and concomitant desmoglein titres and acetylcholine receptor antibody titres was estimated.

Results:

The mean age of our patient was 46yrs old and the male : female ratio was 1:2.2. Our study showed female predominance in all the age groups. Most common subtype of pemphigus was mucocutaneous type. Majority of the patients had mild disease at the time of presentation. The clinical phenotype of pemphigus patients correlated with desmoglein antibody profile i.e the mean value of Dsg3 was higher in mucosal and mucocutaneous subtype of pemphigus and had statistical significance of p-value of 0.02. There was predominance of high titres of Dsg3 in the study population. There was statistically significant correlation between Dsg1 and Dsg3 antibody titre with PDAI scoring (p-value =<0.01) Acetylcholine receptor antibody titre was positive in 22% of our patients. But showed no statistical significance on correlation with Dsg1, Dsg3 and PDAI.

Conclusion:

Desmoglein 1 and desmoglein 3 antibody titre correlated well with PDAI clinical scoring at the time of presentation and hence can be used as a tool for the diagnosis of pemphigus. Though 17 patients were positive for AChRAb yet there was no statistical significant correlation of AChRAb with PDAI, anti Dsg1 and anti Dsg3 titre. Hence

further study on a large sample and on newer cholinergic receptors is recommended to prove its usefulness in the diagnosis and disease activity correlation.

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ANNEXURE-1

Information sheet

Explanation of the purpose of research?
Pemphigus is a common disease in our population. In a patient with this disease the patient's body produces certain chemicals called antibodies which react against certain molecules called desmogleins. In addition to this it is recently detected that antibodies to acetylcholine receptors on keratinocytes are also involved in the disease process. The level of these antibodies are measured by laboratory methods using ELISA technique. There are several clinical scoring systems described to assess the extent and severity of the disease in the patient. There are visual scoring system which are scored by the doctors by looking at the extent and type of lesions present on the skin. You are requested to participate in a study to help us decide whether the antibodies against acetylcholine receptors correlate well with disease severity as measured by PDAI score and also compared with desmoglein antibody titre. We hope to include around 80 patients from this hospital in this study.

What will you have to do if you participate in his study?
If you agree to participate in this study once you have been diagnosed to have pemphigus you will be requested to allow a doctor to visualize and score the extent and severity of the skin involvement using the PDAI scoring. You are also required to give blood sample for the measurement of concomitant serum antiacetylcholine receptor antibody level. The detected serum antibody level is then correlated with disease severity and compared with desmoglein antibody titre which is routinely measured in pemphigus patients

Can you withdraw from this study after it starts?
Your participation in this study is entirely voluntary and you are also free to decide to withdraw permission to participate in this study. If you do so, this will not affect your usual treatment at this hospital in anyway.

What will happen if you develop any study related injury?
We do not expect any injury to happen to you but if you do develop any side effects or problems due to the study these will be treated at no cost to you.

Will you have to pay for the blood test?
The test anti acetylcholine receptor antibody will be done for you free of cost.

Will your personal details be kept confidential?
The results of this study will be published in a medical journal but you will not be identified by name in any publication or presentation of results. However your medical notes may be reviewed by people associated with the study without your additional permission should you decide to participate in this study.

If you have any queries contact Dr.Rosemary at (0416 2282054) or e.mail to dr.rosy@gmail.com

Informed Consent form to participate in a research study

Study Title: Antibodies to Acetylcholine receptors and disease activity in pemphigus

Study Number: _____

Subject's Initials: _____ Subject's Name: _____

Date of Birth / Age: _____

(Subject)

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

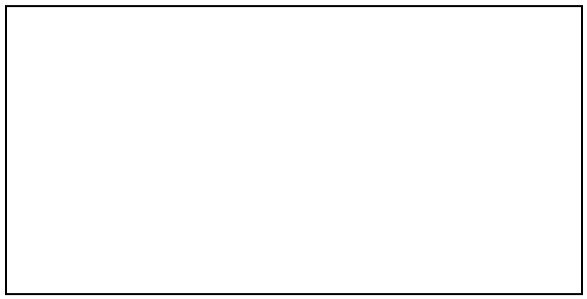
(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable

Date: _____/_____/_____

Signatory's Name: _____ Signature:

Or



Representative: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

ANNEXURE-2

PROFORMA FOR PATIENTS

Sl number:

Name:

Hospital number:

Age: Sex:

Address:

Diagnosis:

Presenting complaints:

Type of lesion	*Duration*	*Site*
Blisters on skin		
Oral lesions		

Past history of disease:

Rx taken:

Drug	*Maximum dose*	*Current dose*	*Duration*
Steroids:			
Oral			
Topical			
Injection			
Azathioprine			
Cyclophosphamide			
Cyclosporin			
MMF			
Others			

Investigations:

Tzanck *Histology* *DIF*

PDAI scores Anatomical location	Activity Erosion/blister or new erythema 0- Absent 1- 1-3 lesions,upto one>2cm in any Diameter,none>6cm 2- 2-3 lesions, atleast 2>2cm, None >6cm 3- >3 lesions, none >6cm 5 - >3 lesions, and or atleast One >6cm 10 - >3 lesions and or atleast One >16cm or entire area	Damage Post inflammatory hyperpigmentation or erythema from resolv No: of lesions <3 0- absent 1- present
Ears		
Nose		
Face		
Neck		
Chest		
Abdomen		
Back/buttocks		
Arms		
Hands		
Legs		
Feet Genitalia		

Total skin	/120	/12
Scalp	0 – Absent 1 – 1 quadrant 2 – 2 quadrant 3 – 3quadrant 4 – whole 10 – one lesion >6cm	
Total	/10	/1
Mucosa	0 – Absent	
	1 – 1 lesions	
	2 – 2-3 lesions	
	5 - >3lesion or 2 lesion >2cm	
	10 – entire area	
Eyes		
Nose		
Buccal mucosa		
Hard palate		
Soft palate		
Upper gingiva		
Lower gingiva		
Tongue		
Floor of mouth		
Labial mucosa		
Posterior pharynx		
Anogenital		
Total activity score	/120	Total damage score /13

First visit	Date	AChRAb	Dsg-3Ab	Dsg-1Ab

ANNEXURE -3

Glossary to master sheet

1) S.No. – Serial number
2) Date – Date of inclusion into the study
3) Name – Name of the patient
4) Hosno. – Hospital number of the patient
5) Age – Age of the patient
6) Sex – Sex of the patient 1- male, 2- female
7) ADD - Address of the patient 1- TN , 2-kerala, 3-karnataka, 4-andhra Pradesh, 5 – jharkand, 6- bihar, 7- West Bengal, 8 – Bangladesh, 9 – Assam, 10 - Orissa
8) BlisterskinDur – Duration of the blisters on the skin
9) Blister skin Site – Site of onset of blister on the skin

   Face -1, chest – 2, abdomen – 3, 4 – arm, 5 – scalp, 6 – back, 7 - neck
10) OralDur – Duration of the oral lesion
11) Oralsite – Site of onset of oral lesion

    Lip – 1, gingiva – 2, buccal mucosa -3, tongue - 4
12) Diag – diagnosis of the patient

    Pemphigus vulgaris – 1, others – 0
13) Subtype - subtype of pemphigus vulgaris

    Mucosal -1, cutaneous – 2, mucocutaneous -3
14) Treat – Treatment taken at the time of presentation
15) Oster – oral steroid taken or not?
16) Namesteroid – name of the steroid

Prednisolone -1, dexamethasone -2

17) Maxidose – maximum dose of the steroid in mg taken during the course of the disease

18) Currentd – current dose of the steroid in mg

19) steroidDur – duration of the steroid treatment in the patient in months

20) tster – application of topical steroid

21) Az – treated with azoran?

22) Maxidoseaz – maximum dose of azoran used by the patient

23) Currentdoseaz – current dose of azoran in mg in the patient

24) Durationaz – duration of treatment with azoran in the patient in mg.

25) Cycl – treated with cyclophosphamide ?

26) Maxidosecycl – maximum dose of cyclophosphamide used by the patient in mg

27) Currentdosecycl – current dose of cyclophosphamide taken by the patient

28) Durcycl – duration of treatment with cyclophosphamide

29) Others – treated with other drugs? 1- mycophenolate mofetil, 2- dapsone, 3-intravenous immunoglobulin

30) Maxidoseoth – maximum dose of other drugs used in mg

31) Current doseoth – current dose of other drug used in the patient in mg

32) Durationoth – duration of treatment with other drug

33) Tz – tzanck – acantholytic cells seen? Yes or No

34) DIF – direct immunofluroscence showing fishnet positivity – yes or no

35) Hp - histopathology showing suprabasal bulla – yes or no

36) PDAISK – Total pemphigus disease area index score in the skin

37) PDAIMU – total PDAI score in the mucosa

38) PDAISC – total PDAI score in th scalp

39) PDAIDSC – total damage score

40) PDAITSCR – total PDAI score of skin , mucosa , scalp and damage score

41) Achrabcod – serum acetycholine receptor antibody titre in nanomoles/ml

42) Dsg3A – serum anti desmoglein -1 antibody titre in units/l

43) Dsg5A – serum anti desmoglein antibody titre in u/l

ANNEXURE -4

Master sheet

sno	date	hosno	age	sex	blisterski	blistersk1	oraldur	oralsite	diag	Subtype0	treat	oster	namesteroi	maxidose	currentd
1	31/10/2014	084460g	31	2	12	2	24	3	1	3	TRUE	TRUE	1	20	20
2	18/11/2014	914556f	79	1	3	2	2	3	1	3	TRUE	TRUE	1	40	40
3	22/11/2014	438982c	30	1	24	5	24	3	1	3	TRUE	TRUE	1	40	15
4	22/11/2014	099338g	40	2	18	2	96	1	1	3	TRUE	TRUE	1	40	15
5	01/12/2014	077626g	45	2	96	4			1	2	TRUE	TRUE	1	15	15
6	12/12/2014	115585g	49	2	96	7	12	3	1	3	TRUE	TRUE	1	20	20
7	15/12/2014	936255f	56	2	1	6	1	3	1	3	FALSE	FALSE			
8	16/12/2014	887883f	43	1			36	3	1	1	TRUE	TRUE	1	70	10
9	18/01/2014	514748a	51	2	60	3	60	4	1	3	TRUE	TRUE	1	40	15
10	17/12/2014	618363d	29	2	0		24	2	1	1	TRUE	TRUE	1	20	7.5
11	20/12/2014	330784d	34	2	84	6	84	3	1	3	TRUE	TRUE	1	40	10
12	18/12/2015	110953c	49	2	60	5	12	3	1	3	TRUE	TRUE	1	60	20
13	23/12/2014	640488c	41	2	24	2	1	3	1	3	TRUE	TRUE	1	60	5
14	31/12/2014	124961g	47	1	1	3	3	3	1	3	TRUE	TRUE	1	40	20
15	06/06/2015	133511g	64	1	12	2	12	3	1	3	TRUE	TRUE	1	60	45
16	26/01/2015	144890g	46	1	3	5	42	3	1	3	TRUE	TRUE	1	40	20
17	30/12/2014	915641f	22	2	6	2	2	3	1	3	TRUE	TRUE	1	75	30
18	06/02/2015	945670f	52	2	1	2	1	3	1	3	FALSE	FALSE			
19	19/02/2015	146665f	64	2	48	3	48	3	1	3	TRUE	TRUE	1	70	10
20	21/02/2015	767003f	52	1	72	2	36	3	1	3	TRUE	TRUE	1	40	20
21	29/01/2015	445722b	42	2	36	6			1	2	TRUE	TRUE	1	30	12.5
22	05/03/2015	902040f	38	2	24	2	48	3	1	3	TRUE	TRUE	1	80	12.5
23	07/03/2015	156159f	23	2	36	2	36	3	1	3	TRUE	TRUE	1	60	30
24	12/01/2015	423250b	34	1	96	1			1	2	TRUE	TRUE	1	30	5
25	09/03/2015	010237d	56	2	96	2	12	3	0		TRUE	FALSE			
26	31/03/2015	192916g	50	1	12	2			0		TRUE	TRUE	1	50	10
27	07/04/2015	313092c	35	2	120	2	210	3	1	3	TRUE	TRUE	1	45	0
28	08/04/2015	198558g	50	2	9	1	1	3	1	3	TRUE	TRUE	1	75	30
29	19/03/2015	184343g	58	2	18	5	18	3	1	3	TRUE	TRUE	1	45	7.5
30	09/04/2015	792388d	70	2	0		60	3	1	1	TRUE	TRUE	1	50	5
31	28/02/2015	829628d	56	2	24	2	24	3	1	3	TRUE	FALSE			
32	18/04/2015	265015d	52	2			108	3	1	1	TRUE	TRUE	1	35	0
33	21/04/2015	321054b		2	120	2	24	3	1	3	TRUE	TRUE	1	60	10
34	21/04/2015	816530d	26	2	60	5	60	2	1	3	TRUE	TRUE	1	50	0
35	22/04/2015	426672f	57	2	24	2	24	3	1	3	TRUE	TRUE	1	60	10
36	23/04/2015	124590c	61	2	36	2	36	3	1	3	TRUE	TRUE	1	60	5
37	25/04/2015	339562f	23	2	48	2	36	3	1	3	TRUE	TRUE	1	40	12.5
38	30/04/2015	622276f	32	1	0		24	3	1	1	TRUE	TRUE	1	60	10
39	30/04/2015	795403f	59	1	36	2	36	3	1	3	TRUE	TRUE	1	70	5
40	06/08/2015	224971f	42	2	48	5	48	3	1	3	TRUE	TRUE	1	60	10
41	13/05/2015	9555326	29	2	1	6	6	3	1	3	TRUE	TRUE	1	60	0
42	19/05/2015	894476b	67	2	24	5	36	3	1	3	TRUE	TRUE	1	60	0
43	19/05/2015	254620f	55	1	0		36	3	1	1	TRUE	TRUE	1	50	20
44	26/05/2015	179648g	55	2	9	3	1	3	1	3	TRUE	TRUE	1	40	30
45	30/05/2015	539683d	54	1	72	6	72	3	1	3	TRUE	TRUE	1	80	5
46	22/01/2015	142793g	70	1	3	6	3	3	1	3	TRUE	TRUE	1	20	20
47	04/06/2015	754074f	33	1	36	6	36	3	1	3	TRUE	TRUE	1	60	15
48	03/06/2015	181917g	42	2	0		12	3	1	1	FALSE	FALSE			
49	10/06/2015	241376g	22	2	0		12	3	1	1	TRUE	TRUE	1	20	20
50	22/06/2015	244496g	61	1	0		3	3	1	1	TRUE	TRUE	1	60	20
51	20/06/2015	244068g	24	2	0		6	3	1	1	TRUE	FALSE			
52	23/06/2015	247941g	67	1	7	2	24	3	1	3	TRUE	TRUE	1	10	10
53	23/06/2015	198747f	46	2	36	2	36	3	1	3	TRUE	TRUE	1	45	5
54	25/06/2015	241237g	32	2	4	2	4	3	1	3	TRUE	TRUE	1	10	10
55	25/06/2015	253552g	27	2	24	5	24	3	1	3	TRUE	TRUE	1	30	20
56	25/06/2015	251578g	56	2	12	4	12	3	1	3	TRUE	TRUE	1	20	20

57	26/06/2015	767219d	41	2	6	2			1	2	TRUE	TRUE	1	40	5
58	01/07/2015	256957g	41	1	12	2	12	3	1	3	TRUE	TRUE	1	20	5
59	02/07/2015	659616f	57	2	0		24	3	1	1	TRUE	TRUE	1	40	10
60	07/07/2015	835669b	39	2	12	2	3	3	1	3	TRUE	TRUE	1	30	20
61	26/02/2015	266130f	63	1	36	5	36	3	1	3	TRUE	TRUE	1	50	7.5
62	08/07/2015	628588d	50	2	0		60	3	1	1	TRUE	TRUE	1	40	10
63	08/07/2015	263230g	31	1	4	2	1	3	1	3	TRUE	TRUE	1	40	20
64	08/07/2015	264820g	42	2	1	3	1	3	1	3	FALSE	FALSE			
65	13/07/2013	965602f	33	2	12	2	12	3	1	3	TRUE	TRUE	1	60	20
66	14/07/2014	923554d	63	1	36	3	36	3	1	3	TRUE	TRUE	1	50	0
67	28/07/2015	279207g	48	2	6	3	0		1	2	FALSE	FALSE			
68	01/08/2015	156113d	53	2	120	2	120	3	1	3	TRUE	TRUE	1	35	20
69	01/08/2015	281705g	48	2	0		36	3	1	1	TRUE	TRUE	1	20	20
70	04/08/2015	411096d	67	1	24	2	6	3	1	3	TRUE	TRUE	1	25	25
71	18/08/2015	818617f	49	1	1	6	12	3	1	1	TRUE	TRUE	1	60	10
72	11/07/2015	262492g	45	2	0		48	3	1	1	TRUE	TRUE	1	40	10
73	11/07/2015	054772g	46	2	10	2	10	3	1	3	TRUE	TRUE	1	50	10
74	21/03/2015	598048c	45	2	120	4	120	3	1	3	TRUE	TRUE	1	60	0
75	25/07/2015	277165g	36	2	4	2	4	3	1	3	TRUE	TRUE	1	30	30
76	05/03/2015	570869c	53	2	60	2	60	3	1	3	TRUE	TRUE	1	80	10
77	12/02/2015	377156c	68	1	120	2	120	3	1	3	TRUE	TRUE	1	45	10

steroiddur	tster	Az	maxidoseaz	currentdos	duraz	cycl	maxidosecy	currentdo1	durcycl	others	maxidoseot	currentdo2	duroth
12	FALSE	TRUE	75	75	12	FALSE							
1	FALSE	TRUE	50	50	1	FALSE							
24	FALSE	TRUE	75	75	24	FALSE							
96	FALSE	TRUE	100	50	5	FALSE							
96	FALSE	FALSE				FALSE							
84	FALSE	TRUE	100	50	48	FALSE							
	FALSE	FALSE				FALSE							
12	FALSE	FALSE				FALSE							
24	FALSE	FALSE				FALSE							
24	FALSE	TRUE	75	75	24	FALSE							
24	FALSE	TRUE	100	100	24	FALSE							
60	FALSE	TRUE	100	100	60	FALSE							
24	FALSE	FALSE				FALSE							
3	FALSE	FALSE				FALSE							
6	FALSE	FALSE				FALSE							
24	FALSE	TRUE	100	50	12	FALSE							
1	FALSE	FALSE				FALSE							
	FALSE	FALSE				FALSE							
48	FALSE	FALSE				FALSE							
24	FALSE	TRUE	75	75	12	FALSE							
36	FALSE	TRUE	50	50	24	FALSE							
12	FALSE	TRUE	75	75	12	FALSE							
36	FALSE	TRUE	75	75	12	FALSE							
72	FALSE	TRUE	150	100	60	FALSE							
	FALSE	TRUE	50	50	6	FALSE							
12	FALSE	TRUE	100	50	6	FALSE							
60	FALSE	TRUE	50	50	2	FALSE							
1	FALSE	FALSE				FALSE							
1	FALSE	FALSE				FALSE							
60	FALSE	TRUE	100	100	60	FALSE							
	FALSE	TRUE	75	75	24	FALSE							
108	FALSE	TRUE	75	0	60	FALSE							
120	FALSE	TRUE	50	50	36	FALSE							
36	FALSE	TRUE	50	50	24	FALSE							
24	FALSE	TRUE	150	150	12	FALSE							
36	FALSE	FALSE				FALSE							
36	FALSE	TRUE	50	50	24	FALSE							
24	FALSE	FALSE				FALSE							
36	FALSE	TRUE	100	75	12	FALSE							

48	FALSE	FALSE				FALSE				1	2000	2000	48
2	FALSE	TRUE	50	0	2	FALSE							
24	FALSE	TRUE	50	0	24	FALSE							
36	FALSE	TRUE	100	100	36	FALSE							
1	FALSE	FALSE				FALSE							
72	FALSE	TRUE	50	50	72	FALSE							
2	FALSE	FALSE				FALSE							
36	FALSE	TRUE	50	50	12	FALSE							
	FALSE	FALSE				FALSE							
2	FALSE	FALSE				FALSE				2	100	100	1
2	FALSE	FALSE				FALSE							
	TRUE	FALSE				FALSE							
6	FALSE	TRUE	50	50	1	FALSE							
36	TRUE	FALSE	50	50	12	FALSE							
2	FALSE	FALSE				FALSE							
24	FALSE	FALSE				FALSE							
1	FALSE	TRUE	50	50	12	FALSE				2	100	100	1
6	FALSE	TRUE	100	100	6	FALSE							
2	FALSE	TRUE	50	50	8	FALSE							
24	FALSE	TRUE	100	100	12	FALSE							
3	FALSE	FALSE				FALSE							
36	FALSE	TRUE	75	75	24	FALSE							
60	FALSE	TRUE	50	50	48	FALSE							
1	FALSE	FALSE				FALSE							
	FALSE	FALSE				FALSE							
12	FALSE	TRUE	25	25	12	TRUE	50	50	12				
36	FALSE	TRUE	100	50	6	FALSE							
	FALSE	FALSE				FALSE							
120	FALSE	TRUE	50	25	36	FALSE							
12	FALSE	TRUE	50	50	12	FALSE							
6	FALSE	FALSE				FALSE				2	100	100	24
12	FALSE	TRUE	125	125	24	FALSE							
1	FALSE	TRUE	50	50	12	FALSE							
3	FALSE	FALSE				FALSE							
36	FALSE	TRUE	75	75	24	FALSE							
4	FALSE	TRUE	50	50	4	FALSE							
36	FALSE	TRUE	75	75	36	FALSE							
120	FALSE	TRUE	50	50	12	FALSE							

tz	hp	dif	pdaisk	pdaimu	pdaisc	pdaidsc	pdaitscr	achrabcod	dsg3a	dsg5a
TRUE	TRUE	TRUE	2	3	0	5	10	2.1	172	5.7
TRUE	TRUE	TRUE	11	0	0	4	15	3	71	197
TRUE	TRUE	TRUE	0	2	2	0	4	0.2	190	78.3
TRUE	TRUE	TRUE	24	6	4	4	38	1.1	187	147
TRUE	TRUE	TRUE	2	0	0	6	8	1	2	160
TRUE	TRUE	TRUE	3	0	3	5	11	3.1	165	68
TRUE	TRUE	TRUE	31	6	10	0	46	1.1	207	79
TRUE	TRUE	TRUE	0	13	0	0	13	3.1	183	20
TRUE	TRUE	TRUE	0	2	0	2	4	2	150	2
TRUE	TRUE	TRUE	0	7	0	0	7	0.2	46	10.1
TRUE	TRUE	TRUE	0	1	0	2	3	0.2	2	3
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	173	13
TRUE	TRUE	TRUE	6	0	0	3	9	0.2	48.2	34.1
TRUE	TRUE	TRUE	10	7	0	0	17	1.1	181	124
TRUE	TRUE	TRUE	0	0	0	3	3	3	69.5	2
TRUE	TRUE	TRUE	13	12	4	0	29	0.2	220	63.3
TRUE	TRUE	TRUE	0	0	0	3	3	0.2	17.7	3.9
TRUE	TRUE	TRUE	17	6	2	0	23	0.2		
TRUE	TRUE	TRUE	8	0	0	0	8	0.2	134	153
TRUE	TRUE	TRUE	0	0	6	0	6	0.2	169	28.8
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	65	118
TRUE	TRUE	TRUE	0	0	0	3	3	0.2	56.8	2.6

TRUE	TRUE	TRUE	0	0	0	0	0	1.2	61	59
TRUE	TRUE	TRUE	6	0	0	3	9	0.5	9.4	16.7
TRUE	TRUE	TRUE	12	22	0	1	35	0.2	161	9.2
TRUE	FALSE	TRUE	8	0	1	0	9	0.2	2	143
TRUE	TRUE	TRUE	0	10	0	0	10	0.2	184	56
TRUE	TRUE	TRUE	13	21	4	4	42	0.2	184	197
TRUE	TRUE	TRUE	5	6	1	0	12	0.2	192	179
TRUE	TRUE	TRUE	0	0	0	0	0	2.1	31	2.6
TRUE	TRUE	TRUE	0	0	0	0	0	3	25	2
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	66.8	2.5
TRUE	TRUE	TRUE	0	2	0	0	2	0.2	4.5	56
TRUE	TRUE	TRUE	0	0	2	0	2	0.2	2	228
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	167	2
TRUE	TRUE	TRUE	0	2	0	0	2	0.2	202	21.9
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	135	16.6
TRUE	TRUE	TRUE	0	5	0	0	5	0.2	178	49.5
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	196	19.8
TRUE	TRUE	TRUE	0	1	0	0	1	0.2	2.2	15
TRUE	TRUE	TRUE	47	15	10	3	75	0.2	173	151
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	129	71.7
TRUE	TRUE	TRUE	0	16	0	0	17	0.2	154	24
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	135.9	15.5
TRUE	TRUE	TRUE	0	0	1	0	1	3.2	166	2
TRUE	TRUE	TRUE	2	0	2	0	4	0.2	69	122
TRUE	TRUE	TRUE	0	0	2	0	2	0.2	2	2
TRUE	FALSE	TRUE	0	18	0	0	18	0.2	145	2.5
TRUE	FALSE	TRUE	0	20	0	0	20	0.2	199	71.4
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	149.5	7.5
TRUE	FALSE	TRUE	0	19	0	0	19	0.2	171	174
TRUE	TRUE	TRUE	13	0	3	2	18	0.2	132	189
TRUE	TRUE	TRUE	0	0	0	0	0	4	180	3.5
TRUE	TRUE	TRUE	5	19	0	3	27	0.2	154	89.8
TRUE	TRUE	TRUE	8	0	2	2	12	0.2	141	159
TRUE	TRUE	TRUE	12	6	2	5	25	0.2	164	120
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	86.3	21
TRUE	TRUE	TRUE	15	2	0	3	20	0.2	77.2	69
TRUE	TRUE	TRUE	0	0	2	0	2	0.2	2.9	10.8
TRUE	TRUE	TRUE	0	0	7	0	7	0.2	148	9.8
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	109	10.4
TRUE	TRUE	TRUE	0	19	0	0	19	0.2	160.5	2
TRUE	TRUE	TRUE	13	6	2	0	21	0.5	192.1	166
TRUE	TRUE	TRUE	10	10	3	0	23	0.2	192.9	141
TRUE	TRUE	TRUE	8	8	3	6	25	0.2	162.9	126.4
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	9.6	9.7
TRUE	TRUE	TRUE	4	2	2	2	10	0.2	83.1	181
TRUE	TRUE	TRUE	2	6	0	0	8	0.2	161	11.9
TRUE	TRUE	TRUE	0	15	0	0	15	0.2	164.2	188
TRUE	TRUE	TRUE	9	6	0	5	20	0.2	201.8	183.5
TRUE	TRUE	TRUE	0	1	1	0	2	0.2	92	234
TRUE	TRUE	TRUE	0	10	0	0	10	0.2	162.6	11.6
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	2.3	7
TRUE	TRUE	TRUE	1	2	0	0	3	0.2	97	111
TRUE	FALSE	TRUE	6	8	2	3	19	0.2	89	29.1
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	54	46.4
TRUE	TRUE	TRUE	0	0	0	0	0	0.2	100	3.9